Universidade do Minho
Escola de Ciências da Saúde

Mário Jorge Alves de Oliveira

The programming effects of antenatal exposure to corticosteroids in the brain

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Tese de Doutoramento em Medicina
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Professor Doutor Nuno Sousa

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DECLARAÇÃO

Nome: Mário Jorge Alves de Oliveira

Endereço electró nico: mario.oliv@gmail.com

Telefone: +351 915303901

Número de Cartão do Cidadão : 11 229930

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Aos meus pais

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Mário Oliveira

Abstract

Up to 7-10% of the pregnant women receive at least one dose of synthetic glucocorticoids (GC) in order to promote fetal lung maturation and, despite animal and human data suggesting growth restriction and other deleterious effects on the developing brain and other organs, the use of repeated doses still occurs in clinical practice. Moreover, it is established that early life adverse events can shape physical and mental health in adulthood. In fact, there is accumulating evidence that exposure to stress or elevated levels of GC during crucial stages of development contributes for the appearance of neurospyschiatric conditions, such as anxiety and depression.

In the present work, we assessed the impact of exposure to dexamethasone (DEX), a synthetic GC widely used in clinics, during gestational days 18-19 on the adult progeny. Following an initial endocrine and behavioral characterization, a neuroanatomical assessment of brain areas implicated in anxiety and fear conditioning, the bed nucleus of stria terminalis (BNST) and amygdala, respectively, was carried out; in these brain regions molecular and neurochemical assessments were also performed. In parallel, and since brain sexual differentiation begins during late gestation, we also aimed to evaluate the implications of antenatal DEX exposure on adult male sexual behavior and to establish possible molecular and neurochemical correlates, namely on the nucleus accumbens (NAcc) and the hypothalamus. To assess the differential the impact of the activation of glucocorticoid or mineralocorticoid receptors, whenever possible, we also included an additional group exposed to a natural GC at the same gestational period.

Our data showed that prenatal DEX triggers a hyperanxious phenotype and alters fear behavior in adulthood. These behavioral traits correlated with hypothalamus-pituitary-adrenal (HPA) axis hyperresponsiveness and increased volume of the BNST, particularly the anteromedial

subdivision, whose bipolar neurons displayed increased dendritic length; in addition, we also found increased expression of the synaptic plasticity-related genes synapsin and NCAM in this brain region. Notably, the effects of antenatal DEX exposure were opposite in the amygdala, which displayed reduced volume due to dendritic atrophy. Despite the absence of differences in dopamine (DA) and its metabolite levels in the BNST, the levels of this neurotransmitter were substantially reduced in the amygdala, where an up-regulation of the DA receptor 2 was also found.

Regarding the impact of prenatal DEX on adult male sexual behavior, decreased number and increased mounts and intromissions latencies were observed. Reduced serum testosterone and increased hypothalamic expression of the androgen receptor (AR) correlated with these changes; additionally, reduced DA levels and increased dopamine receptors expression were observed in both hypothalamus and NAcc. Interestingly, exposure to equipotent doses of corticosterone, a natural corticosteroid, resulted in milder phenotypical changes.

In summary, our data support that in utero DEX exposure differentially modulates anxiety and fear behavior through distinct morphological, neurochemical and molecular changes. Moreover, adult male sexual behavior is also affected through modification of specific neuronal and endocrine mediators. Importantly, a less detrimental phenotype seems to result from exposure to equipotent doses of natural GC, which points to the mediating role of mineralocorticoid and glucocorticoid receptors in these processes and calls for a reappraisal and judicious use of these drugs in the clinics.

Resumo

Cerca de 7-10% das mulheres grávidas recebem, pelo menos, uma dose de glucocorticóides (GC) sintéticos para acelerar a maturação pulmonar fetal e, apesar de estudos em animais e humanos apontarem para atraso de crescimento e outros efeitos nefastos no cérebro e outros órgãos em desenvolvimento, o recurso a várias doses persiste na clínica. É reconhecido que eventos adversos durante fases precoces da vida podem moldar a saúde física e mental do adulto. De facto, existe evidência crescente de que a exposição a stress ou níveis elevados de GC durante etapas cruciais do desenvolvimento contribuem para o aparecimento de distúrbios neuropsiquiátricos, como ansiedade e depressão.

No presente trabalho, avaliámos o impacto da exposição a dexametasona (DEX), um GC sintético amplamente utilizado na prática clínica, durante os dias de gestação 18-19, na descendência adulta. Após caracterização endócrina e comportamental, procedeu-se à avaliação neuroanatómica de áreas cerebrais implicadas na ansiedade e medo condicionado, o núcleo da estria terminal (NET) e a amígdala, respectivamente; também se efectuaram análises moleculares e neuroquímicas nestas regiões. Paralelamente, e uma vez que a diferenciação sexual cerebral se inicia durante a fase final da gravidez, quisemos também avaliar as consequências da exposição pré-natal a DEX no comportamento sexual masculino e estabelecer possíveis correlações moleculares e neuroquímicas, nomeadamente no núcleo accumbens (NAcc) e hipotálamo. De modo a avaliar o diferente impacto decorrente da activação do receptores glucocorticóides ou mineralocorticóides, incluímos, quando possível, um grupo experimental adicional exposto a GC naturais durante o mesmo período.

Os nossos dados revelaram que a DEX pré-natal desencadeia um fenótipo de hiperansiedade e altera o comportamento relacionado com o medo na idade adulta. Estes traços comportamentais

correlacionaram-se com uma resposta exacerbada do eixo hipotálamo-pituitária-suprarrenal (HPS) e um aumento do volume do NET, particularmente da divisão anteromedial, cujos neurónios bipolares exibiam um aumento do comprimento dendrítico; nesta região cerebral também se observou aumento da expressão dos genes relacionados com a plasticidade sináptica, sinapsina e molécula de adesão celular neuronal (MACN). De destacar que os efeitos da exposição pré-natal a DEX foram opostos a nível da amígdala, com redução de volume devido a atrofia dendrítica. Apesar de não se terem encontrado diferenças nos níveis de dopamina (DA) e respectivos metabolitos no NET, os níveis deste neurotransmissor encontravam-se consideravelmente reduzidos na amígdala, onde se observou um aumento do receptor 2 da DA.

Relativamente ao impacto da DEX pré-natal no comportamento sexual masculino do adulto, observou-se uma redução do número e aumento da latência para cópula com ou sem penetração. Estas alterações correlacionaram-se com diminuição da testosterona sérica e aumento da expressão hipotalámica do receptor dos androgénios (RA); observou-se ainda redução dos níveis de DA e aumento da expressão dos receptores da dopamina no hipotálamo e no NAcc. De realçar que a exposição a doses equipotentes de corticosterona, um corticosteróide natural, originou alterações fenotípicas mais ténues.

Em resumo, os nossos dados sugerem que a exposição intra-uterina a DEX modula de forma diferente a ansiedade e o medo através de distintas alterações morfológicas, neuroquímicas e moleculares. Também o comportamento sexual masculino do adulto é afectado através da modificação de mediadores neuronais e endócrinos específicos. De destacar que, da exposição a doses equipotentes de GC naturais, parece resultar um fenótipo menos afectado, o que aponta para o papel mediador dos receptores mineralocorticóides e glucocorticóides nestes processos e reclama para a necessidade de uma reavaliação e uso sensato destas substâncias na prática clínica.

CONTENTS

ABBREVIATIONS LIST

ACTH – adrenocorticotrophic hormone

AR – androgen receptors

ASR – acoustic startle reflex

AVP – arginine-vasopressin

BLa – basolateral anterior amygdaloid nucleus

BNST – bed nucleus of the stria terminalis

BNSTam – anteromedial division of the bed nucleus of the stria terminalis

CeA – central amygdaloyd nucleus

COA – cortical amygdalar nucleus

CORT – corticosterone

CRH – corticotrophin-releasing hormone

DEX – dexamethasone

Drd1/Drd2 - dopamine D1 and D2 receptors

GABA – gama-amino-butiric-acid

GC – glucocorticoids

GR – glucocorticoid receptor

EPM – elevated plus maze

FPS – fear-potentiated startle

HPA – hypothalamus-pituitary-adrenal

HPLC – high performance liquid chromatography

MeA – medial amygdaloid nucleus

MR – mineralocorticoid receptor

NAcc – nucleus accumbens

NCAM – neural cell adhesion molecule

NLOT – nucleus of the lateral olfactory tract

PPI – prepulse inhibition

PVN – paraventricular nucleus of the hypothalamus

Chapter 1

Introduction

1. INTRODUCTION

1.1 The clinical application of corticosteroids during late gestation

Corticosteroids are involved in the development and maturation of several fetal organs (Miller, 1998). These processes may be hastened by exogenous administration of these hormones, typically used to promote lung maturation in preterm labours (Crane et al., 2003). Liggins first described the major benefit of exogenous glucocorticoids (GC) administration in the prevention of the respiratory distress syndrome in premature infants (Liggins and Howie, 1972). The benefits of these drugs, whose efficiency is present through a wide range of gestational ages (24 to 34 weeks), comprise the reduction of mortality, respiratory distress syndrome, and intraventricular hemorrhage (NIH, 1994). Thus, in clinical practice, the prescription of corticosteroids became a common recommendation during late pregnancy, when at risk of premature labour (NIH, 1995). In Europe and North America, it is estimated that up to 7-10% of the pregnant women receive at least one dose of synthetic GC in order to promote fetal lung maturation (Matthews et al., 2004). Antenatal dexamethasone is also an option in the management of congenital adrenal hyperplasia, protecting female fetuses from virilization (Riepe, 2011), though in more prolonged therapeutic schemes.

In daily practice, the use of repeated doses of prenatal glucocorticoids is frequent, despite data showing growth restriction and other deleterious effects on the developing brain and other organs (Newnham and Jobe, 2009). Clinical guidelines on antenatal corticotherapy recommend administration of a total of 24mg of beta- or dexamethasone in a course of two or four doses (Miracle et al., 2008); however, multiple courses of GC are still often administered (Brocklehurst et al., 1999), regardless of the concerns on its potential deleterious effects. A consensus by the

National Institutes of Health Consensus Panel recommended the restriction of the use of repeated GC courses for controlled trial studies and highlighted the need of animal studies in order to assess its potential risks and benefits to the fetus (NIH, 2001).

In fact, a correlation between GC exposure during early life and several metabolic (Barker, 1995; Lindsay et al., 1996), emotional and mood disorders in both animal models and humans in adulthood is now established (Weinstock, 2001; Mesquita et al., 2009; Appendix 3), stressing the need to fully clarify its effects and avoid unnecessary treatments.

1.2 Hypothalamus-pituitary-adrenal (HPA) axis and the developing brain

1.2.1 The HPA axis

The hypothalamus-pituitary-adrenal (HPA) axis plays a major role in the stress response (Sapolsky et al., 1986), which aims to ease adaptation and restore homeostasis. In brief, stress sensitive neurosecretory neurons from the medial parvocellular division of the hypothalamic paraventricular nucleus (PVN), synthesize corticotrophin-releasing hormone (CRH) and arginine-vasopressin (AVP) among other secretagogues, which are released into hypophysial portal circulation and stimulate adrenocorticotrophic hormone (ACTH) production and release to circulation by anterior pituitary corticotropes (Whitnall, 1993). At the adrenal cortex, ACTH promotes glucocorticoid production and release (Figure 1).

The activation of the PVN may be triggered through (1) systemic stressors, that exert their effect through brainstem cathecolaminergic projections (Sawchenko et al., 1996), or (2) processive stressors, which

require higher order sensory processing in limbic circuits. The major projection converging in the PVN arises from the bed nucleus of stria terminalis (BNST), preoptic area and other hypothalamic sites (Herman and Cullinan, 1997). In particular, it is known that the BNST projections to the PVN are modulated by inhibitory GABAergic afferents from central and medial nuclei of the amygdala (Herman et al., 2005) and excitatory CRH projection from the central nuclei of the amygdala and glutamatergic afferents from prefrontal cortex (PFC) and ventral subiculum (Cullinan et al., 1993) (Figure 2).

Following release into systemic circulation, GCs exert a wide spectrum of effects that are critical in stress adaptative response. These include cardiovascular activation (hypertension and positive inotropic effect), suppression of immunity and inflammation, but also metabolic actions (lipolysis, proteolysis, and gluconeogenesis), and effects on the reproductive system (Munck et al., 1984; Sapolsky et al., 2000). To control these effects, a mechanism of negative feedback through which corticosteroids act in the PVN and anterior pituitary gland, regulates their production and thus avoids the deleterious effects of chronic exposure to high levels of corticosteroids (De Kloet and Reul, 1987).

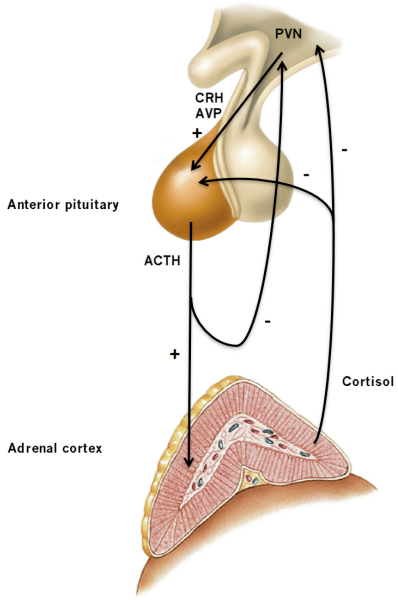

Figure 1. Illustration of the HPA axis circuitry and feedback mechanisms of regulation.

1.2.2 Corticosteroid receptors

Glucocorticoids exert their effects in the brain by binding to two types of nuclear receptors, the type 1 or mineralocorticoid receptor (MR) and the type 2 or glucocorticoid receptor (GR) (McEwen et al., 1986; De Kloet and Reul, 1987). While natural corticosteroids (cortisol in the human and corticosterone in the rat) bind to both despite displaying a greater affinity to MR (10-fold higher), synthetic corticosteroids (such as DEX or betamethasone) bind almost exclusively to GR (Reul et al., 1987).
Concerning brain distribution of corticosteroid receptors, while MR are restricted predominantly to the lateral septum and hippocampus, GR have a more widespread distribution, with a higher density in lateral septum, dentate gyrus, nucleus of tractus solitarius and central amygdala, but also PVN and locus coeruleus (Reul and de Kloet, 1985).
Studies on mature brains have shown opposing actions of MR and GR on neuronal survival in the dentate gyrus. While GR exclusive occupation results in permanent neuronal damage, MR does not (Sousa and Almeida, 2002). Moreover, a protective role has been attributed to MR occupancy, since simultaneous binding to both types of receptors resulted in less deleterious effects (Hassan et al., 1996). Of notice, there is also evidence of hippocampal neuronal death following prenatal administration of DEX (Uno et al., 1990).

1.2.3 Accessibility of GCs to the fetus and fetal brain

Corticosteroids are involved in the development and maturation of several fetal organ systems including the brain, lungs and liver (Miller, 1998). In the same way as most fetal tissues, the placenta expresses glucocorticoid receptors as from mid-gestation (Johnson et al., 2008). The endogenous production of corticosteroids by the mesoderm derived

zona fasciculata/reticularis of the adrenal cortex (cortisol in the human and corticosterone in the rat) occurs late in gestation (Mesiano and Jaffe, 1997; Wotus et al., 1998). Nonetheless, maternal corticosteroids can access the fetal circulation (Dalle and Delost, 1979), with up to 80-90% suffering metabolism by 11b-hydroxysteroid dehydrogenase type 2 (11b-HSD2), during passage through the placenta (Murphy et al., 1974; Gitau et al., 2001).

In contrast to natural corticosteroids which display high affinity to the corticosteroid binding globulin (CBG), synthetic corticosteroids, which are poor substrates for the 11b-HSD2, can also cross the placenta but remain unbound (Cooke et al., 1996), being less amenable to buffering. Additionally, corticosteroids, including dexamethasone can cross the blood brain barrier and access the brain (McEwen et al., 1976; Meijer et al., 1998). Of notice, previous data showed that 11b-HSD2 is highly expressed in the fetal brain during mid-gestation, protecting the developing brain from activation of GR (Diaz et al., 1998). Afterward, levels decline in most brain areas, allowing neuronal and glial maturational events induced by corticosteroids.

1.2.4 Impact of antenatal corticosteroids in neurodevelopment

The *in utero* environment determined by passage of chemical signals across the placenta is crucial to fetal development. Growing evidence suggests that adverse conditions during early life influence growth and brain development and, thus, predispose individuals to mood and anxiety disorders in adulthood (Heim and Nemeroff, 1999; Kaufman and Charney, 2001; Vythilingam et al., 2002). Of relevance for this thesis is the fact that fetal exposure to high levels corticosteroids, either endogenous or exogenous, appears to program the HPA axis and impair mood and emotional behavior in adulthood (Weinstock, 2005). However, the nature of the corticosteroid, the dosage and the period of exposure

are determinant for the outcome (Welberg and Seckl, 2001). Specifically, prenatal exposure to DEX has been shown to increase the messenger RNA (mRNA) levels of corticotropin releasing hormone (CRH) in the central nucleus of the amygdala (CeA) and of GR and MR in the basolateral division of the amygdala (BLa) (Welberg et al., 2001), while endogenous corticosteroids do not seem to affect the latter (Welberg et al., 2000); importantly, the former triggers at the long-term an overactivation of the HPA. In fact, an impairment in the MR/GR balance has been associated with an hyperactivation of the HPA axis (De Kloet, 2003), which was correlated with the development of depression (Holsboer, 2000; Gold and Chrousos, 2002). In addition, HPA axis hyperactivity was associated with morphologic effects in the hippocampus (Sousa et al., 2000; Sousa and Almeida, 2002), prefrontal cortex (Cerqueira et al., 2007), ventral tegmental area (VTA), nucleus accumbens (NAcc) (Leao et al., 2007; Appendix 1), extended amygdala (Pego et al., 2008), and dorsal striatum (Dias-Ferreira et al., 2009) in several rodent models.

Nonetheless, and despite these compelling evidence of extensive activational effects of glucocorticoids, much less is known on the impact of prenatal short-term exposure to corticosteroids in the neuronal networks controlling emotional and sexual behaviors.

1.3 The neuronal network regulating emotional behavior

The amygdala and the bed nucleus of stria terminalis (BNST) exhibit a significant amount of corticosteroids receptors (De Kloet et al., 1998) and are, thus, possible targets of prenatal GC. The knowledge on the amygdala has largely evolved in the last two decades. Challenged the concept of a unique entity (Swanson, 1998), the amygdala is known to be related with diverse functions, including emotion, fear, attention and learning. An adjacent nuclear complex, the BNST, has been recognized

in the vicinity of the stria terminalis. Importantly, for the present thesis, a double dissociation between the involvement of the amygdala and BNST in fear and anxiety has been reported (Davis, 1992b, a, 1998, 2006), with the former being largely implicated in fear processing, whereas the BNST is largely related with anxiety behavior.

1.3.1 The amygdala

The term amygdala was initially used to describe an almond shaped nucleus in the inner temporal lobe (Burdach, 1819). Johnston subsequently reviewed its extension and divisions (Johnston, 1923), and the amygdala to be composed by an archaic group of nuclei related to the olfactory system (central, medial and cortical nuclei, and nucleus of the lateral olfactory tract) and a more recent group of nuclei (lateral and basal). More recently, the concept of the "extended amygdala" emerged, following observations on the topographical and functional similarities between central and medial nuclei of the amygdala and the BNST (Alheid and Heimer, 1988; Alheid et al., 1998). New developments were established on the knowledge of the structural and functional organization of the amygdala (Swanson and Petrovich, 1998), suggesting that it is composed of three main regions: (1) a specialized ventromedial extension of the striatum, (2) a caudal olfactory cortex, and (3) a temporal extension of the claustrum.

Besides its topographic relationship, both morphologic and histochemical similitude support the concept that striatal amygdala consists of a specialized extension of the striatum, being formed by the central and medial nuclei, and anterior area (Alheid and Heimer, 1988; Esclapez et al., 1993; McDonald and Augustine, 1993; Sun and Cassell, 1993). Supporting this idea is the fact that the central (CeA) and medial nuclei of the amygdala (MeA) have GABAergic extrinsic projections, in the same way as the adjacent caudate-putamen (Davis et al., 1994).

Conversely, extrinsic projections from the remaining amygdala are presumed to be mainly glutamatergic, supporting its cortical nature.
In contrast to the somatic motor information modulated by dorsal striatum (caudate-putamen), most information processed in the amygdala is relayed by its major output, the CeA, to several autonomic-related centers, namely in the brainstem and hypothalamus in response to emotional stimuli (Hopkins and Holstege, 1978; Rosen et al., 1991; Bandler and Shipley, 1994). The MeA receives its major sensory input from the accessory olfactory bulb and projects massively to the medial preoptic nucleus and other sexually dimorphic hypothalamic nuclei thought to play key roles in mediating steroid sensitive reproductive functions. Sensory input from the vomeronasal organ, transporting pheromonal information, is projected to the accessory olfactory bulb and then relayed in the MeA, which then projects to the BNST and hypothalamic areas related with reproductive function (Scalia and Winans, 1975; Simerly et al., 1989).

The amygdalar olfactory cortical group consists of the nucleus of the lateral olfactory tract (NLOT), the cortical amygdalar nucleus (COA) and the postpiriform and piriform–amygdalar areas (Swanson and Petrovich, 1998). The former two are considered different areas since the NLOT, and anterior and posterolateral COA receive projections from the main olfactory bulb, while the posteromedial COA receives inputs from the accessory olfactory bulb (Scalia and Winans, 1975). Both the postpiriform and piriform-amygdalar areas receive input from the main olfactory bulb and project to the CeA and several amygdalar regions, respectively (Pitkanen et al., 1997).

The temporal extension of the claustrum is formed by the lateral, basal and posterior nuclei. Consisting of the deepest layers of temporal, piriform and frontal cortex, it forms a frontotemporal system (Swanson and Petrovich, 1998), and is separated from the superficial cortical

layers by the amygdalar capsule, a division of the extreme capsule (Swanson, 1998).

While local circuits are mostly constituted by spine-sparse non-pyramidal neurons containing GABA, acetylcholine and a variety of neuropeptides (McDonald and Pearson, 1989), projections to CeA, ventral caudate-putamen, nucleus accumbens, and frontal cortex are assured by glutamatergic pyramidal neurons (McDonald, 1991). Whereas frontal cortex connections probably take part in the awareness of fear and anxiety, projections to the CeA are essential for autonomic and somatic responses to adverse stimuli. On the other hand, outputs to ventral caudate-putamen and nucleus accumbens convey motivational information to motor areas, permitting avoidance or approach in response to aversive or reinforcer related stimuli, respectively.

1.3.2 The bed nucleus of stria terminalis (BNST)

The BNST is one of the relay stations that conveys inputs from stress-sensitive areas of the cortex and limbic system both to the HPA axis (Figure 2) but also to brain stem nuclei implicated in several emotional behaviors (Herman and Cullinan, 1997). Following initial descriptions on the gray matter surrounding the stria terminalis (Ramón y Cajal, 1911), the expression *bed of the stria terminalis* was later used to name a region comprised between the temporal pole caudally and the base of the olfactory peduncle rostrally (including part of the head of the caudate nucleus) (Johnston, 1923).

Subsequent studies using cyto- and chemoarchitectonic criteria both in developing and adult models suggested the division of the BNST into anterior and posterior components, separated by the vertical fibers of

the stria terminalis (Bayer, 1987; Ju and Swanson, 1989; Ju et al., 1989).

The anterior division comprises ventral, dorsal, and lateral areas, each consisting of several nuclei (Dong et al., 2001). The posterior division contains at least five nuclei: principal, interfascicular, transverse, dorsal, and premedullary. The information on cyto- and chemoarchitectonic characteristics permitted a more precise determination of regional boundaries: a) between the anterior and posterior divisions of the BNST; b) among the several nuclei or cell groups in each division, and c) between the BNST and the ventrally adjacent preoptic region.

Based on projection studies, the ventral and dorsal areas of the anterior division have been arranged in a medial group. This anteromedial division receives inputs from both the MeA and CeA, and from main and accessory olfactory system components of the amygdala (Krettek and Price, 1978; Weller and Smith, 1982; Dong et al., 2001; Dong and Swanson, 2006). This division densely projects to hypothalamic regions strongly related with the neuroendocrine system (Cullinan et al., 1993; Moga and Saper, 1994; Spencer et al., 2005).
Conversely, the lateral (or anterolateral) division receives projections from the CeA and amygdalar components of the main olfactory system (Krettek and Price, 1978; Weller and Smith, 1982; Dong et al., 2001) and participates in the expression of ingestive behaviors and homeostasis through projections to areas related with central autonomic control and midbrain structures coordinating visceral and somatic motor responses (Dong and Swanson, 2003, 2004a, 2006). Besides establishing reciprocal projections with MeA and amygdalar components of the accessory olfactory system (Dong et al., 2001), neurons from the posterior division project to hypothalamic areas that modulate reproductive and defensive behaviors (Dong and Swanson, 2004b, 2006).

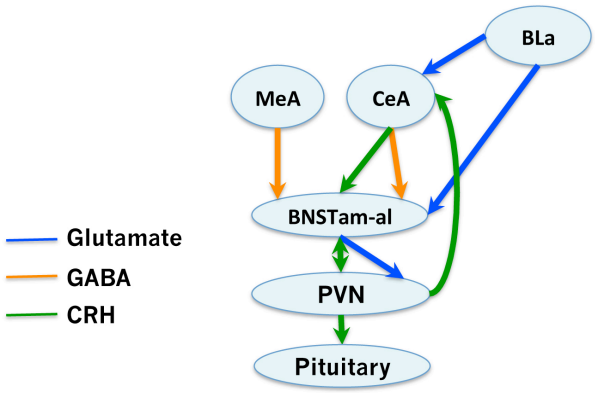

Figure 2. Simplified schematic representation of the extended amygdala circuitry and its modulatory action over the HPA axis.

1.4 Neurobiology of sexual behavior

When describing the patterns of male sexual behavior, motivation to engage in sexual activity and sexual performance may be distinguished. These two aspects of sexual behavior find parallel in the more classic description of appetitive and consummatory behaviors (Ball and Balthazart, 2008). Briefly, there are three major systems regulating sexual motivation and genital and motor responses (Hull et al., 2004): a) the mesolimbic system is critical for appetitive behavior and reinforcement; b) the medial preoptic system contributes to genital reflexes, sexual motivation and motor patterns of copulation; c) the nigrostriatal system enhances the motoric readiness to respond to stimuli (Figure 3).

Dopamine is the common key player in all three systems, easing sexual motivation, copulatory proficiency, and genital reflexes (Giuliano and Allard, 2001). The recognized facilitating effects of DA on male sexual function date from evidence of increased libido and sexual potency as a side-effect in Parkinson patients treated with L-Dopa (Barbeau, 1969; Bowers et al., 1971; Kofman, 1971). More recently, the use of the D1/D2 agonist, apomorphine, to treat erectile dysfunction has achieved

some degree of success (Heaton, 2000; Giuliano and Allard, 2001; Montorsi et al., 2003).

The pathways for sexual excitation involve the activation of incertohypothalamic and mesolimbic dopamine transmission that targets the hypothalamic medial preoptic area (mPOA) and nucleus accumbents (NAcc), respectively (Pfaus, 2009). Conversely, the nigrostriatal dopaminergic pathway, whose cell bodies in the zona compacta of the substantia nigra project to the dorsal striatum (Moore and Looklingland, 1995), has a predominant role in the motor aspects of sexual behavior, as supported by the fact that DA is released only during copulation (Damsma et al., 1992).

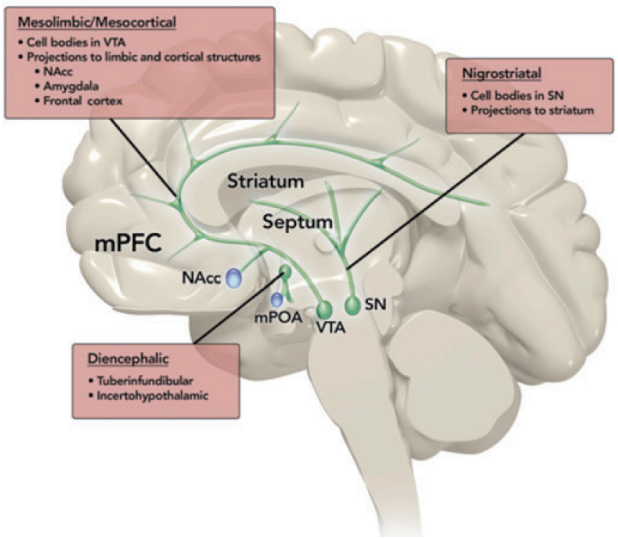

Figure 3. Illustration of brain dopaminergic systems controlling sexual behavior. VTA, ventral tegmental area; mPFC, medial prefrontal cortex; SN, substantia nigra; mPOA, medial preoptic area; NAcc, nucleus accumbens. From Pfaus JG (2009). Pathways of sexual desire. J Sex Med 6:1506-33.

1.4.1 The role of the hypothalamus, the BNST and the amygdala in male sexual behavior

Two cell groups of the mPOA appear to be of particular relevance in the modulation of male sexual behavior in gerbils: the medial and lateral

sexually dimorphic areas, homologous to the rats' medial portion of the medial preoptic nucleus (MPN) and magnocellular MPN, respectively (Yahr, 1995). Diverse mating eliciting sensory stimuli indirectly relay in the mPOA, while reciprocal connections modulate its processing (Simerly and Swanson, 1986). The dopaminergic input to the mPOA originates from the periventricular system, including cell bodies in the medial portion of the mPOA (Simerly and Swanson, 1986; Moore and Looklingland, 1995). In turn, projections from mPOA to other hypothalamic, midbrain, and brainstem nuclei, are known to regulate somatomotor or autonomic patterns and to contribute for sexual motivation (Simerly and Swanson, 1988; Yahr, 1995). It is also important to highlight the efferents to the paraventricular nucleus of the hypothalamus which are critical in the regulation of non-contact erections and copulation.

The modulation of sexual behavior in hypothalamic sites is known to be influenced by hormones, in particular testosterone, and neurotransmitters, namely dopamine. Testosterone is thought to centrally mediate most hormonal effects on male sexual behavior. These hormonal effects are conveyed through androgen receptors present in brain areas relevant for male sexual behavior, including the mPOA or mPOA-anterior hypothalamus (AH) continuum (Yahr, 1995). The effects of testosterone binding may be either long-lasting through promoting protein transcription (McGinnis and Kahn, 1997) or faster, being mediated by membrane receptors affecting ion-channel activity or second-messenger systems (Moore and Evans, 1999; Shakil et al., 2002).

As in other brain regions, two main families of DA receptors in the mPOA differentially affect sexual behavior: a) the D1-like family which includes D1 and D5 subtypes and activate adenylyl-cyclase, and b) the D2-like family which consists of D2, D3 and D4 subtypes and inhibits

the adenylyl-cyclase (Civelli et al., 1993). While it has been shown that an increase in the D2/D1 ratio in the mPOA results in a delay in the onset of copulation and in a reduction of the ejaculatory threshold, possibly by altering autonomic control of penile reflexes (Hull et al., 1989), selective stimulation of D1 receptors increases the rate of copulation and the number of ejaculations (Markowski et al., 1994).

The amygdala and BNST, particularly its posteromedial division, are also of particular relevance in the control of male rodent sexual behavior (Alheid et al., 1995). In the corticomedial region of the amygdala, chemosensory, somatosensory, and hormonal stimuli are integrated and then projected to the mPOA and other central regulatory areas (Hull, 2010). Morevoer, somatosensory afferents from the genitals reach the MeA via the subparafascicular nucleus, which then projects either directly to the mPOA or via the BNST. Interestingly, despite lacking dopaminergic neurons, the MeA seems to facilitate DA release by cellular bodies or terminals in the mPOA, promoting mating behavior (Dominguez and Hull, 2001).

1.4.2 The mesolimbic system

Behavioral data suggest that male rodent sexual behavior is a rewarding and reinforcing behavior. In the mesocorticolimbic circuit, dopaminergic projections from the ventral tegmental area (VTA) to the nucleus accumbens (NAcc) and medial prefrontal cortex (mPFC) play a crucial role in motivation (Alcaro et al., 2007); importantly, for the present context is the fact that DA is released in the NAcc of male rats upon female presentation and during sexual behavior (Pfaus et al., 1990). The disinhibition of dopaminergic projection neurons of the VTA and subsequent release of DA into NAcc is attained following stimulation of mu opioid receptors which block their tonic inhibition by local

GABAergic interneurons (Klitenick et al., 1992; Ikemoto et al., 1997). Interestingly, in prenatally stressed male rats, the absence of copulatory behaviors was associated with a failure to increase extracellular levels of dopamine and its metabolites in the NAcc (Wang et al., 1995). Such data, obtained through simultaneous sexual behavior testing and concomitant microdialysis sampling, suggested that intense environmental stressors might impair NAcc dopamine release (Wang et al., 1995).

A final note, to note that during copulation, in response to the VTA dopaminergic innervation via the mediodorsal thalamus, the mPFC exerts a positive feedback through glutamatergic projections, further promoting the excitation of VTA dopaminergic neurons (Tzschentke, 2000; Balfour et al., 2006). In addition to the VTA, other areas relevant for sexual behavior receive input from the mPFC, namely mPOA, NAcc, and BNST, thus showing that the mPFC also plays a modulatory role in male sexual behavior (Figure 4).

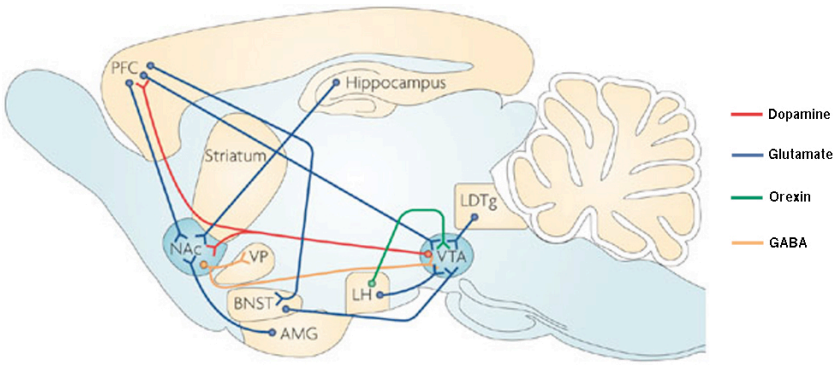

Figure 4. Simplified representation of mesolimbic dopamine system circuitry in the rat brain highlighting the major projections to the nucleus accumbens (NAc) and ventral tegmental area (VTA). AMG, amygdala; BNST, bed nucleus of the stria terminalis; LDTg, laterodorsal tegmental nucleus; LH, lateral hypothalamus; PFC, prefrontal cortex; VP, ventral pallidum. Adapted from Kauer JA, Malenka RC (2007). Synaptic plasticity and addiction. Nat Rev Neurosci 8:844-58.

1.5 Aims of the study

While the use of antenatal GCs triggers long-term gains in several clinical conditions, evidence highlights several undesired “programming effects” that need to be clarified.

The present thesis aims to:

· Characterize the potential effects of brief antenatal exposure to corticosteroids on emotional behavior in adulthood (Chapters 2.1 and 2.2).
· Assess the impact of this prenatal glucocorticoids on the structure of the BNST and amygdala, brain regions implicated in anxiety and fear behaviors, and establish its molecular and neurochemical correlates (Chapter 2.2).
· Establish the impact of antenatal corticosteroids exposure on adult male sexual behavior and on its hormonal and neurochemical mediators (Chapter 2.3).

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Chapter 2

Experimental work

Chapter 2.1

Oliveira M, Bessa JM, Mesquita A, Tavares H, Carvalho A, Silva R, Pêgo JM, Cerqueira JJ, Palha JA, Almeida OF, Sousa N

Induction of a hyperanxious state by antenatal dexamethasone: a case for less detrimental natural corticosteroids

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(2006)

Induction of a Hyperanxious State by Antenatal Dexamethasone: A Case for Less Detrimental Natural Corticosteroids

Mário Oliveira, João M. Bessa, Ana Mesquita, Hugo Tavares, André Carvalho, Rui Silva, José M. Pêgo, João J. Cerqueira, Joana A. Palha, Osborne F.X. Almeida, and Nuno Sousa

Background: *Synthetic glucocorticoids are commonly prescribed during pregnancy, despite a lack of systematic investigations of their potential impact on the developing brain and neurological and behavioral performance.*

Methods: *Neuroendocrine parameters and behavior in the adult offspring of pregnant Wistar rats treated antenatally with either dexamethasone (DEX) or corticosterone (CORT) were monitored; DEX (.1 mg/kg and 1 mg/kg) and CORT (25 mg/kg) were given to pregnant rat dams on gestation days 18 and 19.*

Results: *Despite normal basal levels of corticosterone, the adult offspring of mothers given DEX or CORT displayed abnormal responses in the dexamethasone-suppression test. Neither treatment influenced spatial memory performance, but both DEX and CORT facilitated development of depressionlike behavior following chronic stress. The latter finding demonstrates that high-dose antenatal corticotherapy can impair the organism's resilience to stress in adulthood. Interestingly, comparison of the progeny of CORT-treated and DEX-treated mothers revealed that the latter were more anxious.*

Conclusions: *Since DEX and CORT differ in their affinity for glucocorticoid and mineralocorticoid receptors and corticosteroid-binding globulin, our findings emphasize the need to consider the pharmacologic properties of antenatal corticotherapies and demonstrate the potential long-term benefits of ligands that can bind to both receptors.*

Key Words: Neurodevelopment, corticosteroids, antenatal corticotherapy, anxiety, depression, cognition

Glucocorticoids are prescribed during late gestation in some 10% of risk pregnancies to promote fetal lung maturation (Crane et al 2003; Crowley 1995; National Institutes of Health 1995). Frequently, synthetic glucocorticoids, such as dexamethasone (DEX) or betamethasone, are used rather than cortisol (the natural glucocorticoid in humans) or hydrocortisone (Jobe et al 2003). Chronic antenatal dexamethasone is also the treatment of choice for reducing genital masculinization in female fetuses with congenital adrenal hyperplasia (Forest et al 1998; New et al 2001).

Corticosteroids exert their long-term actions by binding to nuclear receptors whose actions result from the regulation of transcription of corticosteroid-responsive target genes. Two types of receptors, the high-affinity mineralocorticoid receptor (MR) and the low-affinity glucocorticoid receptor (GR), mediate corticosteroid actions in the brain and periphery (Reul et al 1987). Dexamethasone binds exclusively to GR in vivo. In contrast, corticosterone (CORT) (the dominant endogenous glucocorticoid in rodents) binds to both receptors but shows a higher affinity for the MR. Glucocorticoid receptors become activated when endogenous corticosteroid secretion is elevated (e.g., during stress) and chronic GR activation deleteriously affects both peripheral tissues (e.g., bone and muscle atrophy, glucose intolerance) and the brain (Sapolsky 1999). Studies on the mature brain have shown that DEX causes irreversible neuronal damage or even death in the striatum and hippocampus (Almeida et al 2000; Haynes et al 2001; Sousa et al 1998). In contrast, MR occupancy has been reported to confer neuroprotection, and interestingly, concomitant activation of GR and MR by high levels of CORT results in only transient dendritic atrophy and synaptic loss (Sousa and Almeida 2002; Sousa et al 2000). One study in the Rhesus monkey reported the neuronal death-inducing effects of antenatal DEX (Uno et al 1990). Given the relatively common use of glucocorticoid therapy in obstetrics, it seemed prudent to make a systematic reappraisal of the potential harmful effects of antenatal glucocorticoid therapy on the developing brain; additionally, in light of the differing pharmacological profiles of various glucocorticoids, we also considered it worthwhile to compare the effects of different glucocorticoid analogs.

Normal development of the fetal hypothalamic-pituitary-adrenal (HPA) axis is essential for normal growth, organogenesis, and immunity. Recent studies have shown that HPA axis function can be programmed in early life, or even prenatally, by stress, a condition characterized by elevated corticosteroid secretion (Huot et al 2002; Liu et al 2001; Weaver et al 2004). This programming includes permanent alterations to the thresholds for glucocorticoid negative feedback (Bakker et al 1995; Jacobson and Sapolsky 1991; Levitt et al 1996), with resulting basal hypercortisolemia (Huot et al 2002; Levitt et al 1996) and exaggerated HPA responses to subsequent stressors. Besides increasing susceptibility to a number of systemic pathologies such as hypertension, autoimmune disorders, and diabetes (Munck and Guyre 1986; Sapolsky et al 2000), chronic exposure to high levels of glucocorticoids is causally linked to the development of emotional and mood disorders in both experimental animals and humans (Charney and Manji 2004; McEwen 2000).

In this study, either CORT (25 mg/kg) or DEX (Sigma-Aldrich, St. Louis, Missouri) (1 mg/kg or .1 mg/kg) were administered to rat dams in late pregnancy; cognition, emotionality, and susceptibility to display depressionlike behavior were examined in their adult offspring. These measures were complemented by the assessment of HPA axis function, including the efficiency of

From the Neuroscience Group (MO, JMB, AM, HT, AC, RS, JMP, JJC, JAP, NS), Life and Health Science Research Institute, University of Minho, Braga, Portugal; and Neuroadaptations Group (OFXA), Max-Planck Institute of Psychiatry, Munich, Germany.

Address reprint requests to Nuno Jorge Carvalho de Sousa, Life and Health Science Research Institute (ICVS), University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal; E-mail: njcsousa@ecsaude.uminho.pt.

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glucocorticoid negative feedback; impairments of the latter are observed in a significant proportion of patients with major depression (Evans and Nemeroff 1987; Holsboer 1983; Holsboer et al 1995).

Methods and Materials

Animals and Treatments

Pregnant female Wistar rats (Charles River Laboratories, Barcelona, Spain) were individually housed under standard laboratory conditions (light/dark cycle of 12/12 hours, with lights on at 8:00 AM; 22°C ambient temperature; ad libitum food and water). Subcutaneous injections of 1 mg/kg of dexamethasone (DEX1, $n = 4$), 25 mg/kg of corticosterone (CORT, $n = 4$), or saline solution (CONT, $n = 3$) were administered on pregnancy days 18 and 19 (parturition in the rat occurs on pregnancy day 21) (Figure 1). An additional group of pregnant dams ($n = 3$) was treated with a lower dose (.1 mg/kg) of dexamethasone (DEX0.1). Progeny ($n = 8$ per group) were separated and housed according to gender and antenatal treatment on postnatal day 21. Experiments were conducted in accordance with local regulations (European Union Directive 86/609/EEC) and National Institutes of Health (NIH) guidelines on animal care and experimentation.

One major aim of this study was to compare the effects of prenatal exposure to different glucocorticoid receptor ligands (DEX and CORT). Accordingly, drug dosages were chosen to achieve similar transrepressive potencies at the GR (DEX 1 mg/kg vs. CORT 25 mg/kg) (Buttgereit et al 1999; Schimmer and Parker 2001) and the induction of equivalent somatic changes (DEX .1 mg/kg vs. CORT 25 mg/kg). The somatic changes monitored were body weight (at various postnatal intervals), age at eye opening, and thymus and adrenal weights (at sacrifice), as well as various parameters reflecting the activity of the HPA axis (see below).

Neuroendocrine Function Tests

Blood samples were collected by tail venipuncture between 7:00 AM and 9:00 AM when offspring were 2 and 10 months old; additionally, blood samples were collected from offspring aged 2 months for the assessment of basal nighttime serum CORT levels by radioimmunoassay (RIA) (ICN Biomedicals, Costa Mesa, California).

A dexamethasone suppression test (DST) was performed when animals were 10 months old. Briefly, animals were injected subcutaneously with either vehicle or DEX (30 μg/kg) at 9:00 AM and blood samples for CORT determination were obtained at 7:00 PM.

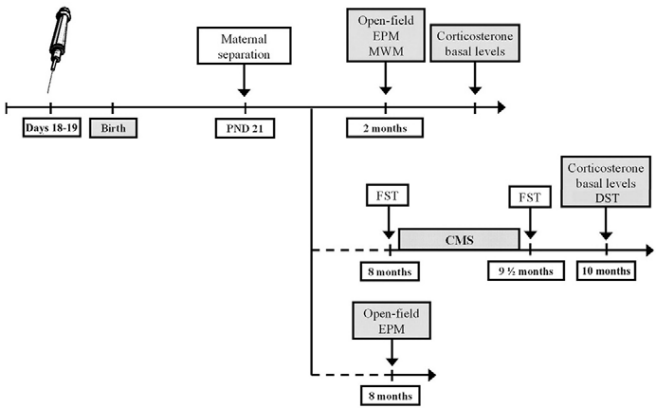

Figure 1. Schematic representation of the experimental design. Subcutaneous injections were administered to pregnant rats at gestation days 18 and 19. Maternal separation was performed at postnatal day 21 (PND21). Elevated plus maze (EPM) and Morris water maze (MWM) tests were performed in two separate sets of animals. In another cohort of animals, the forced swimming test (FST) was performed before or after exposure to chronic mild stress (CMS). Activity of the hypothalamic-pituitary-adrenal (HPA) axis was monitored by assays of basal and stress-induced corticosterone secretion and after the dexamethasone suppression test (DST).

Behavioral Tests

Behavioral evaluation was performed when animals were 2 months old ($n = 8$ per experimental group), and a subgroup of female rats ($n = 6$ per experimental group) was tested at 8 months of age. All tests were conducted during the daily light phase (9:00 AM–6:00 PM).

Open Field

Exploratory behavior was investigated using the open field test in a brightly illuminated (white light) room. Briefly, rats were placed in the center of an arena (43.2 × 43.2 cm; transparent acrylic walls and white floor) (MedAssociates Inc., St. Albans, Vermont) and position was monitored online over a period of 8 minutes with the aid of two 16-beam infrared arrays. Total distances traveled were used as indicators of locomotor activity. Time and distances in the central and predefined peripheral areas were recorded and used to calculate the ratio of time spent in the central quadrant of the platform and the distance traveled in this central area (versus rest of the open field platform). The number and duration of rearings were also recorded.

Elevated Plus Maze

This test was used to assess emotionality (degree of anxiousness); all investigations were carried out under bright white light. Animals were placed (5 minutes) on a black polypropylene plus-shaped platform elevated 72.4 cm above the floor; the maze, consisting of two open arms (50.8 × 10.2 cm) and two closed arms (50.8 × 10.2 × 40.6 cm), was supplied by MedAssociates Inc. The number of entries into each of the arms and the time spent therein were recorded online. Data were processed to yield the ratio of time spent in the open arms versus total time and the number of entries into each arm of the maze.

Morris Water Maze

This maze consisted of a black tank (diameter: 170 cm; depth: 50 cm), divided into quadrants by imaginary lines and filled with water (22°C) to a depth of 31 cm. During testing, a black platform (12 × 12 cm; invisible to the rats) was placed at a height of 30 cm; this platform was placed in a different quadrant of the maze in each test session. The room was dimly lit and extrinsic visual clues were glued to the walls. Data were collected using a video-tracking system (Viewpoint, Champagne au Mont d'Or, France). We used a place-learning task to assess the ability of rats to learn the position of the invisible escape platform. Animals were tested for 5 consecutive days (four trials per day, with a maximum of 2 minutes per trial). The escape platform was placed in the center of an arbitrarily defined quadrant assigned to a specific test subject. To avoid possible bias effects due to geographic preference, "animal-platform position" was paired between the different treatment groups. Test sessions were begun with rats being placed facing the wall of the maze in a defined start position. In the first trial of each day, rats were placed in the quadrant immediately to the right of that containing the escape platform; this procedure was continued in a clockwise

fashion over the subsequent test days. The path described and time spent to reach the platform (latency time) were recorded in the consecutive trials, terminated when the escape platform had been reached; in cases where the escape platform had not been reached within 2 minutes of placing the rat in the maze, the experimenter guided the animal to the platform. In either case, animals were dried and allowed to rest for 30 seconds before being returned to the maze for the remaining test sessions of that day.

Depressionlike Behavior

Learned helplessness, as a measure of susceptibility to depression-related behavior, was assessed in a subgroup of animals using the forced swim test (FST) before and after exposure to a chronic mild stress (CMS) protocol. The first FST was performed in animals aged 8 months; animals were then subjected to CMS for 6 weeks when they were put through a second FST.

The CMS paradigm consisted of a battery of insults based in previously described protocols (Willner et al 1992).

For the FST, rats were placed in cylinders filled with water (25°C) to a depth such that the animals had no solid support for their rear paws. After a 10-minute pretest session, animals were rested for 24 hours before being subjected to the actual tests, which lasted 5 minutes. At the end of each test session, animals were placed on a heating pad (15 minutes) before being returned to their home cages. The cylinders were filled with fresh water after each trial. A video camera placed at the top of the cylinder was used to record test sessions; video recordings were later scored by an investigator blind to the experimental details. Time of immobility (passiveness; defined as time spent either immobile or making righting movements to stay afloat) and latency to immobility were computed.

Data Analysis

Initially, one-way analysis of variance (ANOVA) on ranks (Kruskal-Wallis) was performed to evaluate overall treatment effects on behavioral analysis. Differences between groups were determined by appropriate post hoc pairwise comparisons. Place-learning task performance was analyzed on the average values from blocks of four consecutive trials. A two-factor analysis of learned helplessness in the FST before and after exposure to CMS was performed (considering prenatal exposure × trial), followed by one-way ANOVA to assess between-subject effects. Biometrical data were analyzed by one-way ANOVA, followed by post hoc pairwise comparisons to establish differences between groups. All results are expressed as group means ± SEM. Differences were considered to be statistically significant when $p < .05$.

Results

Antenatal Corticosteroids Affect Birth Weight, Neurodevelopmental Milestones, and HPA Responsiveness in Adulthood

The progeny of animals treated with both DEX and CORT displayed reduced birth weight ($p = .0001$; Table 1); .1 mg/kg of DEX and 25 mg/kg of CORT proved equipotent in this respect. Differences in body weight (CORT-treated and DEX0.1-treated vs. control offspring) were abolished with increasing maturity, whereas body weights in the animals exposed to the high dose (1 mg/kg) of prenatal DEX remained significantly reduced.

In accordance with previous studies (Neal et al 2004), we also observed a trend for an advance in the day of eye opening in the progeny of mothers treated with corticosteroids: postnatal day (PND)13 in the control group versus PND12 in 50% of the high DEX, 37.5% of the low DEX, and 12.5% of the CORT-treated groups.

Basal diurnal and nocturnal corticosterone determinations failed to reveal any significant differences among experimental groups (Figure 2), even though there was a trend for basal nighttime levels of corticosterone to be higher in animals that had been prenatally exposed to DEX at 1 mg/kg. Despite a significant gender effect, with female animals displaying higher corticosterone levels than male animals ($p = .001$), variations among experimental groups were similar between sexes. Importantly, data derived from dexamethasone suppression tests demonstrated an impaired shutoff response in the progeny of both CORT-treated and DEX1-treated animals ($p = .02$).

Progeny of DEX-Treated Mothers Display Reduced Locomotion and Exploratory Activity

Open field testing at 2 months of age demonstrated that several parameters were affected by corticosteroid treatment. The ratio of time spent in central area over total time (Figure 3A) was affected by treatment both in male animals ($H = 12.43$; $p = .006$) and female animals ($H = 10.56$; $p = .014$). The progeny of DEX-treated animals spent proportionately less time in the central area than the control group, both in male animals and female animals. Corticosterone-exposed rats displayed intermediate scores that were not significantly different from control in female animals but differed from DEX groups. This parameter did not differ between animals exposed to 1 versus .1 mg/kg DEX.

Table 1. Body Weight Evolution During the First 60 Days of Life

Treatment	PND 1	PND 10	PND 21	PND 30	PND 60
CONT male animals	6.9 (.2)	30.3 (1.4)	60.9 (1.3)	110.9 (1.0)	267.5 (7.5)
CORT male animals	5.6 (.1)[a]	26.4 (1.5)[b]	56.0 (1.9)[b]	111.8 (4.4)[b]	280.5 (6.6)[b]
DEX 1 mg/kg male animals	5.2 (.2)[a]	20.5 (1.5)[a]	48.4 (3.4)[a]	97.5 (4.3)[a]	234.1 (8.5)[a]
DEX .1 mg/kg male animals	5.6 (.1)[a]	27.3 (.3)[b]	54.0 (.3)[b]	107.1 (1.4)[b]	278.0 (6.3)[b]
CONT female animals	6.7 (.1)	29.3 (1.7)	64.3 (.9)	100.9 (1.4)	186.9 (3.7)
CORT female animals	5.4 (.1)[a]	25.0 (1.4)[a]	59.1 (1.9)[b]	100.4 (2.1)[b]	187.0 (3.9)[b]
DEX 1 mg/kg female animals	5.2 (.1)[a]	20.3 (.9)[a]	48.1 (2.7)[a]	93.4 (2.2)[a]	166.9 (5.0)[a]
DEX .1 mg/kg female animals	5.4 (.1)[a]	23.3 (.2)[a]	58.8 (.8)[b]	98.3 (.9)[b]	184.5 (2.0)[b]

Body weights (g) at PND 1, 10, 21, 30, and 60 of offspring of vehicle (CONT), corticosterone (CORT), and high-dose dexamethasone (DEX; 1 mg/kg) treated pregnant rats. Values represent mean (SEM). Animals exposed to low doses of DEX (.1 mg/kg) do not differ from CORT-exposed animals. PND, postnatal day; CONT, vehicle; CORT, corticosterone; DEX, dexamethasone.

[a]Significantly different from control animals, $p < .05$.

[b]Significantly different from DEX, $p < .05$.

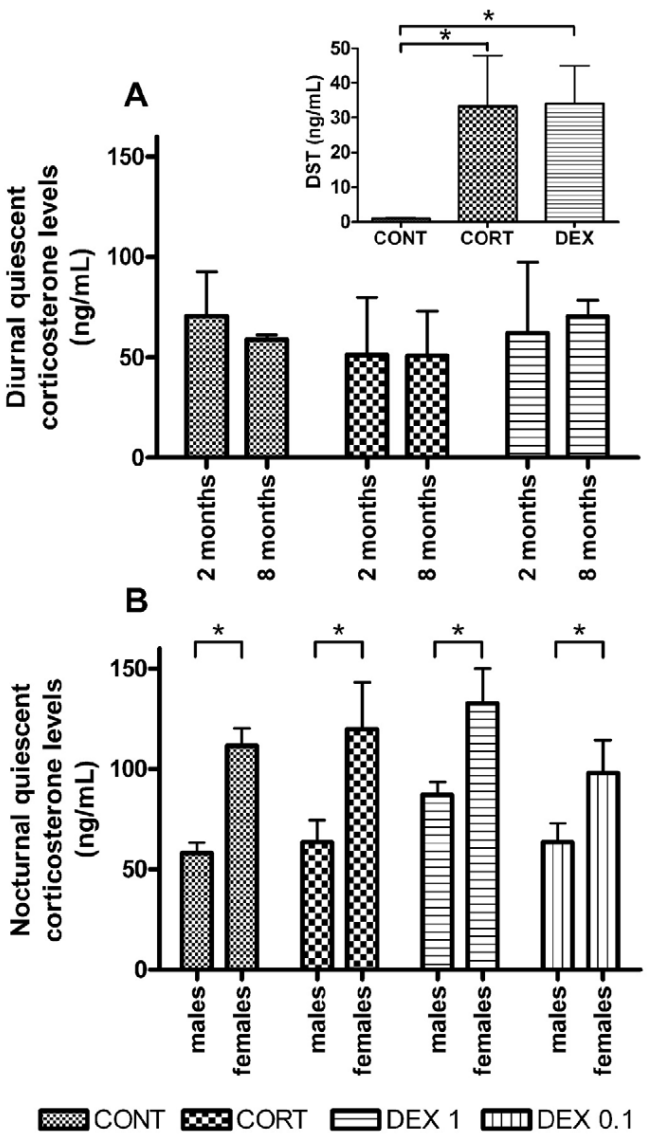

Figure 2. Antenatal corticosteroids affect HPA responsiveness in adulthood. **(A)** Diurnal quiescent levels of corticosterone at 2 and 10 months of age (bottom left) and after the DST (top right) in the female progeny of vehicle- (CONT), corticosterone- (CORT), and dexamethasone- (DEX) injected pregnant rats. **(B)** Nocturnal quiescent levels of corticosterone at 2 months of age in the male and female progeny of vehicle- (CONT), corticosterone- (CORT), high dexamethasone- (DEX1), and low dexamethasone- (DEX0.1) injected pregnant rats. Results are expressed in ng/mL. Error bars represent SEM. The DST results reveal blunted glucocorticoid negative feedback in the offsrping of CORT- and DEX-exposed mothers. *$p < .05$. HPA, hypothalamic-pituitary-adrenal axis; DST, dexamethasone suppression test.

Female animals had significantly higher values than male animals ($p < .001$).

Analysis of total distance traveled (Figure 3B), an index of global locomotor activity, revealed a significant effect of treatment for both male animals and female animals (male animals: H = 18.27, $p = .001$; female animals: H = 11.48, $p = .009$). The male and female offspring of DEX1-treated mothers traveled less distances on the test arena than control animals and CORT. Progeny from DEX0.1-exposed mothers did not differ from DEX1 in male animals, but in female animals the progeny of DEX0.1 traveled longer distances than DEX1. Importantly, DEX0.1 displayed decreased locomotor activity when compared with CORT-exposed rats. Overall, female animals traveled greater distances than male animals ($p = .001$).

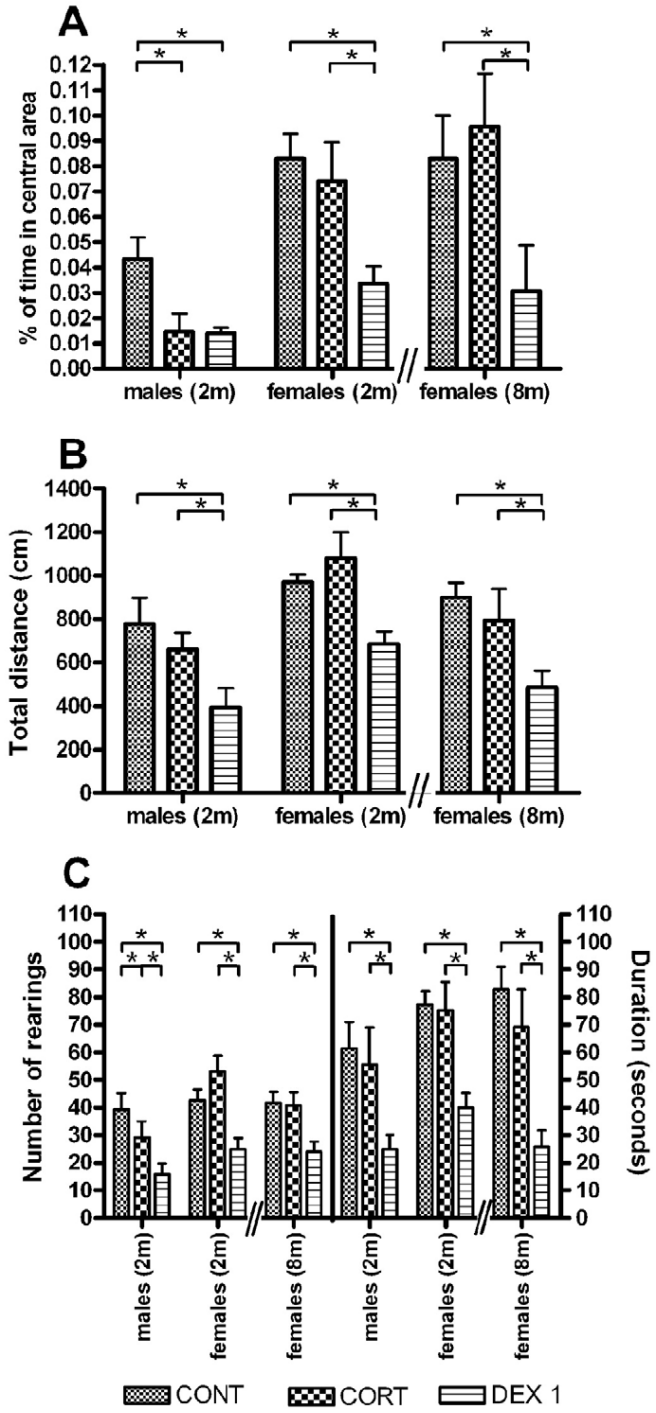

Figure 3. Progeny of DEX-treated mothers display reduced locomotion and exploratory activity. Data collected in the open field test performed at 2 (2m) and 8 months (8m) of age in male and female offspring of vehicle- (CONT), corticosterone- (CORT), and dexamethasone- (DEX) treated pregnant rat dams. **(A)** Percentage of time spent in central area of the open field arena. **(B)** Total distance travelled in centimetres. **(C)** Number (left) and duration (right) of rearings. Error bars represent SEM. *$p < .05$.

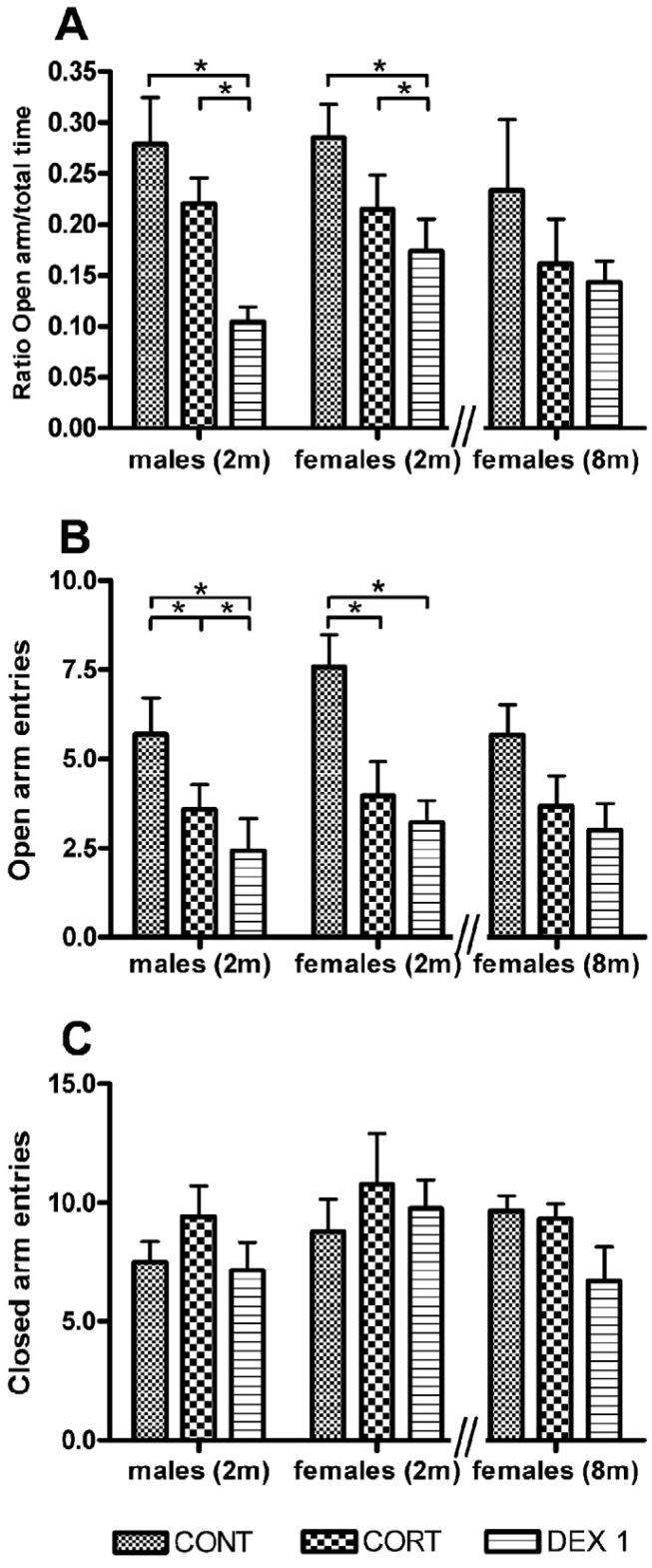

Figure 4. Dexamethasone treatment in late gestation produces a high-anxiety phenotype in adulthood. Results of the elevated plus maze test at 2 (2m) and 8 months (8m) of age in the male and female progeny of vehicle- (CONT), corticosterone- (CORT), and dexamethasone- (DEX) treated pregnant rats. **(A)** Ratio of time spent in open arms over closed arms. **(B)** Number of open arm entries. **(C)** Number of closed arm entries. Error bars represent SEM. *$p < .05$.

Regarding rearing count and duration (Figure 3C), a significant effect of treatment was observed both for male animals (count: H = 8.69, $p = .034$; duration: H = 9.34, $p = .025$) and female animals (count: H = 11.26, $p = .01$; duration: H = 10.94, $p = .012$). On both parameters, results of the male and female progeny of DEX-treated animals were significantly different from those of vehicle- and CORT-treated mothers. The results for the progeny of CORT-treated rats were not significantly different from those found in control animals except in the number of rearings in male animals. Animals exposed to high or low doses of DEX during the antenatal period did not differ on these parameters, but DEX0.1 female animals displayed decreased rearing activity when compared with CORT-treated animals. Duration of rearing was longer in female than in male subjects ($p = .02$).

Data from 8-month-old rats showed that impaired exploratory activity was retained in older rats. In fact, all parameters analyzed were affected by treatment (H = 7.62, $p = .022$; H = 8.24, $p = .016$; H = 7.79, $p = .02$; H = 8.02, $p = .018$, respectively, for percentage of time in central area, total distance traveled, rearing count, and duration). Comparisons between groups revealed that the progeny of DEX-treated animals displayed significantly less time in the center of the arena and decreased locomotion and rearing activity when compared with control animals and CORT-exposed animals.

DEX Treatment in Late Gestation Triggers a High-Anxiety Phenotype in Adulthood

Analysis of variance revealed that the ratio of time spent in open arms (vs. total time) in the elevated plus maze (Figure 4A) was significantly affected by antenatal treatment in both male (H = 18.83, $p = .001$) and female (H = 12,84, $p = .005$) animals. Treatment of mothers with DEX significantly reduced the percentage of time spent in the open arms as compared with control animals and CORT-treated rats in both sexes. Progeny of CORT-treated rats displayed scores that also differed from DEX0.1 in male animals but not in female animals; no significant differences were found between animals treated with high versus low doses of DEX.

Analysis of the number of open arm entries (Figure 4B) revealed a significant effect of treatment in male animals (H = 12.07, $p = .007$) and female animals (H = 16.28, $p = .001$). Offspring of DEX-treated mothers showed a lower number of entries into the open arms compared with control animals. Offspring of CORT-treated dams differed from DEX1 and DEX0.1 in male animals, while DEX0.1 and DEX1 did not differ in both male animals and female animals.

Groups did not differ in the number of closed arm entries in both male animals and female animals (Figure 4C).

Similar behavioral patterns were observed when the offspring of CORT- and DEX-treated mothers were tested at 8 months of age; notably, however, none of the parameters analyzed reached statistical significance.

Spatial Memory Is Not Influenced by Antenatal Corticotherapy

In the place-learning task of the Morris water maze, all animals improved their performance throughout the 5 days of testing (Figures 5A, 5B). Repeated measures analysis of the data on mean escape latency failed to reveal significant differences between any of the experimental groups.

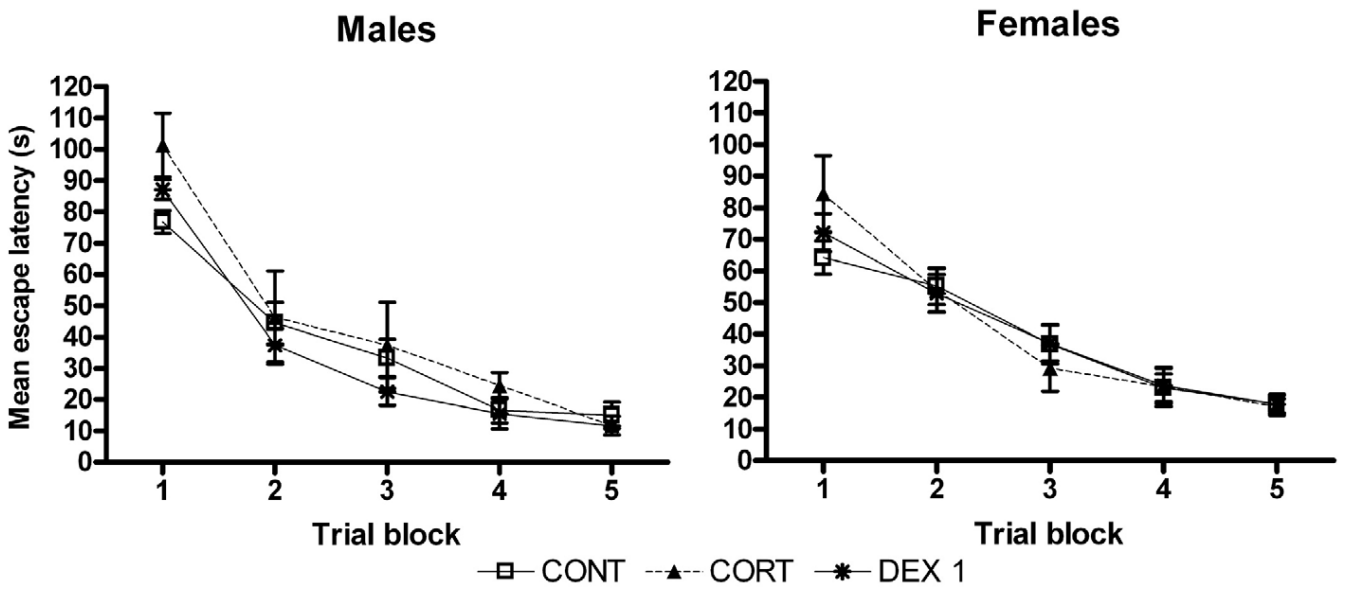

Figure 5. Spatial memory is not influenced by antenatal corticotherapy. Results of the place-learning task on the Morris water maze performed at 2 months of age in male and female offspring of vehicle- (CONT), corticosterone- (CORT), and dexamethasone- (DEX) treated rats on gestation days 18 and 19. Data from each day were grouped and learning analyzed in blocks of trials. Error bars represent SEM.

Antenatal Corticotherapy Increases Susceptibility to Depressivelike Behavior

Duration of immobility and latency to immobility in the FST were significantly affected by the factor "trial number" ($p < .001$, Figure 6A and $p < .001$, Figure 6B, respectively). None of the experimental groups differed on any of the parameters measured when the FST was applied in basal conditions. However, between-group differences became evident when the FST was applied to animals that were previously exposed to the CMS paradigm ($H = 6.63$, $p = .036$ for immobility time and $H = 7.63$, $p = .02$ for latency to immobility): notably, antenatal exposure to either CORT or DEX1 resulted in a significant increase in immobility in the FST, and the latency to display of immobility was also significantly reduced in rats exposed to DEX1 and CORT.

Comparison between the FST before and after exposure to CMS revealed that prenatal treatments with DEX1 and CORT were associated with significantly increased immobility in the FST and decreased latency to display immobility. No significant differences were found in the FST between the CORT and DEX1 groups or between the genders.

Discussion

Fetal development is, in part, determined by the maternal environment through the passage of chemical signals across the placenta. Moreover, in utero experience strongly influences growth and brain development in later life. Of particular relevance to psychiatry is the increasing evidence that adverse conditions during early life can predispose individuals to mood and anxiety disorders in adulthood (Heim and Nemeroff 1999; Kaufman and Charney 2001; Vythilingam et al 2002). The data presented here clearly show that corticosteroid treatment during pregnancy increases anxietylike behavior in adult offspring. They also show that exposure to corticosteroids during prenatal life can program the brain such that it becomes more likely to express depressionlike behavior following exposure to stressful experience. Both the increased anxiety and vulnerability to succumb to lifetime stressors may be related to the fact that antenatal corticosteroids interfere with the mechanisms governing glucocorticoid negative feedback at the neural and/or hypophyseal levels (Charmandari et al 2005; Welberg and Seckl 2001; Weaver et al 2004).

While fetal production of glucocorticoids occurs relatively late in gestation, maternal corticosteroids can cross the placental barrier (Dalle and Delost 1979; Gitau et al 2001). Synthetic corticosteroids such as dexamethasone also have access to the fetus, but unlike their endogenous counterparts, they do not bind to corticosteroid binding globulin (CBG) and, as such, neither the mother nor fetus can buffer their actions. Our findings that prenatal administration of either CORT or DEX results in accelerated eye opening (a measure of neurological development) and retarded body growth are in keeping with the view that corticosteroids can act permissively to accelerate some developmental processes (cf. Gransbergen and Mulder 1998; Neal et al 2004; Slotkin et al 1992), while transiently or permanently delaying others (cf. Gransbergen and Mulder 1998). Interestingly, impeded somatic growth, one of the best-known deleterious effects of antenatal corticosteroid exposure, was here found to be more prominent after antenatal treatment with DEX, as compared with CORT, even when administered at equivalent doses (in terms of gene transrepressive potency). Within the context in which the present work was done, it is important to note that the similarity of the behavioral phenotypes of the high-dose and low-dose DEX-treated animals was greater than that between animals treated with low-dose DEX versus CORT.

Corticosteroids differ in their quality and magnitude of effects according to whether they activate MR, GR, or both of these receptors. In biological assays, DEX displays up to 25-fold greater selectivity for GR as compared with CORT, which elicits both mineralocorticoidlike and glucocorticoidlike effects. Chronic GR activity is associated with numerous peripheral and central pathologies. In contrast, a "yin-yang" relationship between MR and GR is thought to help maintain homeostasis and health. Stress can disrupt the subtle mechanisms governing the MR/GR

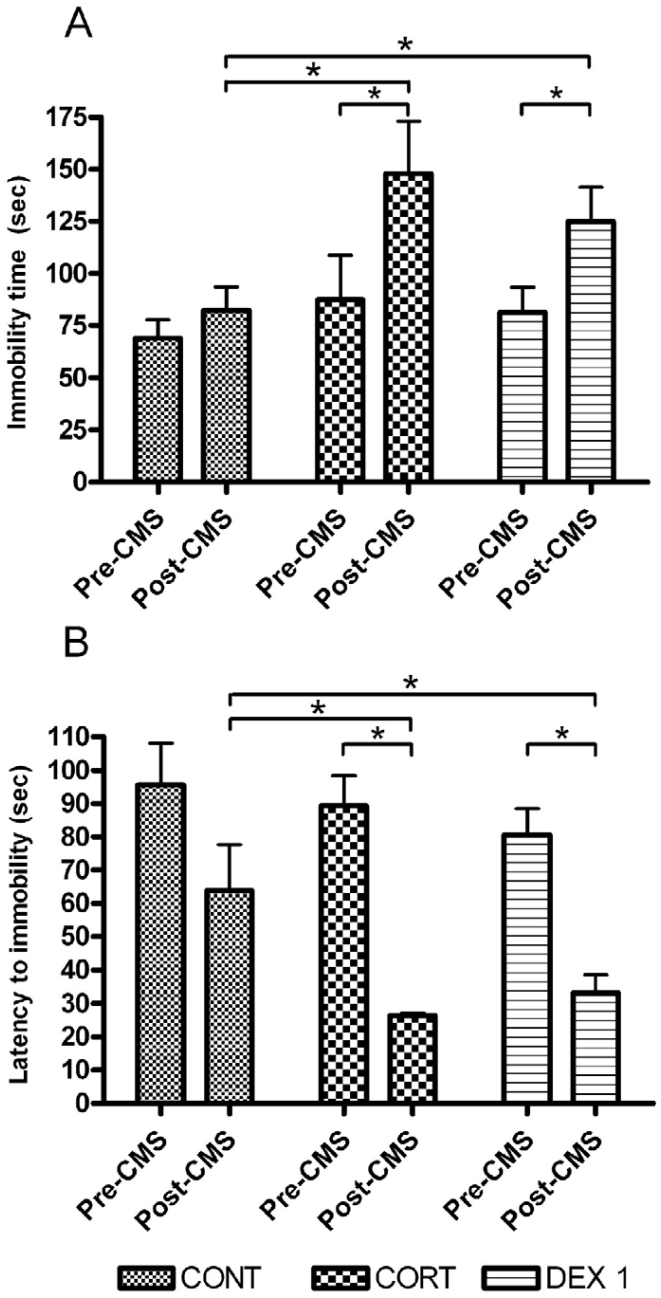

Figure 6. Antenatal corticotheraphy increases susceptibility to depressive-like behavior. Susceptibility to depressivelike behavior in the progeny of vehicle- (CONT), corticosterone- (CORT), and dexamethasone- (DEX) treated pregnant rats was assessed in forced swimming tests before or after exposure to a chronic mild stress (CMS) paradigm. **(A)** Duration of immobility. **(B)** Latency to immobility. Error bars represent SEM. *$p < .05$.

balance and lead to chronic hyperactivity of the HPA axis due to an impairment of glucocorticoid negative feedback mechanisms (De Kloet 2003). Hypersecretion of corticosteroids is strongly linked with depressive illness (Gold and Chrousos 2002; Holsboer 2000). The corticosteroid hypothesis of mood disorders is centered on the finding of aberrant responses to the DST or DEX/CRH tests (Evans and Nemeroff 1987; Holsboer 2000) in a sizeable proportion of patients suffering from major depression. Increased anxiety can predispose an individual to develop depression, and it is therefore interesting to note that impaired glucocorticoid feedback also seems to underlie anxiety disorders (Reul and Holsboer 2002; Strohle and Holsboer 2003).

The present observations that antenatal DEX, but not CORT, produces persistent effects on emotionality are important in light of the above-mentioned associations between the HPA axis, emotionality, and mood. On the other hand, although control, antenatal CORT-treated, and DEX-treated animals showed equal rates of acquisition of learned helplessness (FST), we observed that animals treated during prenatal life with either CORT or DEX were more susceptible to the development of depressionlike symptoms (performance in FST) if they had additionally been exposed to chronic mild stress. Interestingly, animals exposed to antenatal CORT and DEX also showed similar impairments in the DST.

How can the similar responses of the CORT and DEX groups in the DST and FST (after stress) be reconciled with their differing emotional states? One potential explanation may be found in the fact that anxiety and depression result from changes in distinct neurochemical and anatomical substrates; each of these substrates are likely to display differing sensitivity to corticosteroids, reflecting their differing rates of development and relative expression of GR and/or MR (Jutapakdeegul et al 2003; McCormick et al 2000; Owen and Matthews 2003; Rosenfeld et al 1993). Thus, brain structures governing emotionality (primarily, the amygdala) versus spatial memory (primarily, the hippocampus) may be subject to differential programming by corticosteroids during specific time windows (cf. Brabham et al 2000; Weinstock 1997). In addition, the possibility that individual brain areas may only become vulnerable after certain thresholds (magnitude and/or duration of hypercortisolism) have been reached should be considered.

The pronounced differences between the emotional states of animals exposed to antenatal CORT versus DEX deserve consideration of the likely underlying neurobiological mechanisms. The hyperanxiety induced by antenatal DEX points to the amygdala (or its regulatory circuits) as a key target(s) of this GR-selective corticosteroid. Concordant with this, it is pertinent to note that DEX has been previously shown to increase the messenger RNA (mRNA) levels of corticotropin releasing hormone (CRH) in the central nucleus of the amygdala (CeA) and of GR and MR in the basolateral division of the amygdala (BLA) (Welberg et al 2001), while endogenous corticosteroids do not seem to affect the latter (Welberg et al 2000).

Sex-specific neuroendocrine alterations in response to the prenatal corticosteroid milieu, as shown by Dean and Matthews (1999), may also indirectly impact on the expression of emotional and cognitive behaviors. The basal corticosterone secretion profiles in animals exposed to antenatal corticosteroids confirmed previous studies showing that such antenatal treatments do not interfere with gender differences in HPA axis activity (Kitay 1961; Critchlow et al 1963). Interestingly, gender did not prove to be a significant factor in any of the behavioral measurements in the present study. However, we observed that changes in emotional behavior were generally more pronounced in female animals, in agreement with a previous report that the behavioral effects of prenatal stress are more profound in female subjects (Bowman et al 2004). Thus, it is important to consider cyclic changes in ovarian activity in future work on this issue, especially in light of a report showing that corticosteroid-induced prenatal programming includes effects on ovarian cyclicity (Liu et al 2001) and the fact that there are profound male-female

differences in anxiety-like behavior under basal conditions (cf. Mitev et al 2003).

The present study demonstrates that prenatal exposure to glucocorticoids leads to long-lasting effects on brain function and that these effects vary according to the pharmacological profile of the drug used. Interestingly, while exposure to DEX and CORT do not differ in their ability to influence cognitive processes and susceptibility to stress-induced depressionlike behavior, animals exposed to antenatal DEX show a hyperanxious phenotype. Although caution should be exerted in extrapolating these findings in the rat to humans, they provide an impetus for clinical investigations on this topic, given that glucocorticoids are usually administered during equivalent (late) phases of human pregnancy (Jobe 2003). We propose that cortisol/hydrocortisone might be a better alternative to DEX therapy during pregnancy. This view is bolstered by reports showing that cortisol (1 g/kg) has the same therapeutic efficacy as DEX and other closely related potent GR agonists such as betamethasone during pregnancy (Crowley 1995) and neonatal life (Watterberg 2004). The fact that DEX (unlike natural corticosteroids) does not bind to CBG allows this glucocorticoid to easily cross the placenta; thus, the lack of endogenous buffering capacity most likely contributes to the programming of hyperemotionality by antenatal DEX.

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Chapter 2.2

Oliveira M, Rodrigues AJ, Leão P, Cardona D, Pêgo JM, Sousa N

The bed nucleus of stria terminalis and the amygdala as targets of antenatal glucocorticoids: implications for fear and anxiety responses

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ORIGINAL INVESTIGATION

The bed nucleus of stria terminalis and the amygdala as targets of antenatal glucocorticoids: implications for fear and anxiety responses

Mário Oliveira · Ana-João Rodrigues · Pedro Leão · Diana Cardona · José Miguel Pêgo · Nuno Sousa

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Abstract
Rationale Several human and experimental studies have shown that early life adverse events can shape physical and mental health in adulthood. Stress or elevated levels of glucocorticoids (GCs) during critical periods of development seem to contribute for the appearance of neuropsychiatric conditions such as anxiety and depression, albeit the underlying mechanisms remain to be fully elucidated.
Objectives The aim of the present study was to determine the long-term effect of prenatal exposure to dexamethasone- DEX (synthetic GC widely used in clinics) in fear and anxious behavior and identify the neurochemical, morphological and molecular correlates.
Results Prenatal exposure to DEX triggers a hyperanxious phenotype and altered fear behavior in adulthood. These behavioral traits were correlated with increased volume of the bed nucleus of the stria terminalis (BNST), particularly the anteromedial subdivision which presented increased dendritic length; in parallel, we found an increased expression of synapsin and NCAM in the BNST of these animals. Remarkably, DEX effects were opposite in the amygdala, as this region presented reduced volume due to significant dendritic atrophy. Albeit no differences were found in dopamine and its metabolite levels in the BNST, this neurotransmitter was substantially reduced in the amygdala, which also presented an up-regulation of dopamine receptor 2.
Conclusions Altogether, our results show that in utero DEX exposure can modulate anxiety and fear behavior in parallel with significant morphological, neurochemical and molecular changes; importantly, GCs seem to differentially affect distinct brain regions involved in this type of behaviors.

Keywords Anxiety · Fear · Amygdala · Glucocorticoid · Prenatal · BNST · Stereology · Neurodevelopment · Corticosteroids · Dopamine

Introduction

Since the initial evidence on its benefit in the prevention of respiratory distress syndrome in preterm infants (Liggins and Howie 1972), antenatal glucocorticoids (GCs) have gained wide clinical use in the enhancement of fetal lung maturation in pregnancies at risk of preterm delivery (Crane et al. 2003; Crowley 1995; NIH 1995). In fact, accumulating evidence shows a substantial decrease in neonatal morbidity and mortality, which strongly supported the recommendations for its clinical use (Hofmeyr 2009; NIH 1995). Additionally, antenatal GCs have been use to treat other conditions such as congenital adrenal hyperplasia (Speiser et al. 2010).

GCs act predominantly through intracellular receptors which control the transcription of GC-responsive target genes (Matthews 2001). While endogenous GCs (cortico-

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M. Oliveira · A.-J. Rodrigues · P. Leão · D. Cardona · J. M. Pêgo · N. Sousa (✉)
Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Campus de Gualtar, 4710-057, Braga, Portugal
e-mail: njcsousa@ecsaude.uminho.pt

M. Oliveira · A.-J. Rodrigues · P. Leão · D. Cardona · J. M. Pêgo · N. Sousa
ICVS/3B's-PT Government Associate Laboratory, Braga/Guimarães<<<, Portugal

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sterone in rats and cortisone in humans), at basal levels, display higher affinity to the mineralocorticoid receptors (MR), leaving glucocorticoid receptors (GR) receptors largely unoccupied (De Kloet et al. 1998), synthetic GCs such as dexamethasone (DEX) can readily cross the placenta and bind almost exclusively to GR (Reul et al. 1987). GRs can also be activated when endogenous GC secretion is elevated and this chronic activation is noxious for both the periphery and the brain (Sapolsky 1999). One relevant finding is that despite both being efficacious in preventing respiratory distress syndrome (Crowley 2000), synthetic GCs effects seem to be more deleterious than endogenous GCs (Oliveira et al. 2006).

While the use of antenatal GCs triggers long-term gains in several clinical conditions, evidence is also revealing the detrimental effects of antenatal exposure to these hormones; in fact, it is now established a correlation between GC exposure in early life with several metabolic (Barker 1995; Lindsay et al. 1996) and emotional and mood disorders in both animal models and humans in adulthood (Mesquita et al. 2009; Weinstock 2001). These so-called "programming effects" of GCs may result from endocrine, morphological, neurochemical, and molecular changes in specific brain regions; as examples, we have shown that antenatal exposure to DEX significantly changes the mesolimbic circuit (Leao et al. 2007) while others have shown deleterious effects in the prefrontal cortex (Diaz Heijtz et al. 2010), amygdala and hypothalamus (Nagano et al. 2008). Importantly, previous studies from our lab have shown that antenatal exposure to GCs induces a hyperemotional phenotype in adulthood (Oliveira et al. 2006). Given the established role of the amygdala and bed nucleus of stria terminalis (BNST) in fear and anxiety (Davis 1992a, b, 1998, 2006), herein we decided to perform an extensive morphological and molecular analysis of these brain regions in adult animals exposed to antenatal DEX and to search for correlations with fear and anxiety behaviors.

Materials and methods

Animals and treatments

Experiments were conducted in accordance with European Union regulations (Directive 86/609/EEC) and National Institutes of Health guidelines on animal care and experimentation.

Fourteen pregnant Wistar rats (Charles River Laboratories, Barcelona, Spain) were individually housed under standard laboratory conditions (room temperature 22°C; 12/12 h light/dark cycle; food and water ad libitum). Subcutaneous injections of dexamethasone (DEX, 1 mg/kg; $N=7$) or vehicle (CONT, 1 mL/kg; $N=7$) were administered on embryonic (ED) 18 and 19 of pregnancy, as previously described (Oliveira et al. 2006; Oliveira et al. 2011). Weaning was carried out at postnatal day 21; male offspring was randomly distributed in groups of two animals per cage according to treatment. Ten males from 4 to 5 different litters of each treatment were tested for behavior at 3 months age.

Elevated plus maze

The elevated plus maze test, used to assess the degree of anxiousness, was performed on a black polypropylene plus-shaped platform elevated 72.4 cm above the floor (ENV-560; MedAssociates Inc, St Albans, VT, USA). The maze consisted of two open arms (50.8×10.2 cm) and two closed arms (50.8×10.2×40.6 cm). Animals were tested over a period of 5 min, under bright white light. The number of entries into each of the arms and the time spent therein were recorded using a system of infrared photobeams. Data were processed to obtain the ratio of time spent in the open arms versus total time and the number of entries into each arm of the maze.

Acoustic startle as a function of stimulus intensity

Acoustic startle reflex was measured in a startle response system (SR-LAB, San Diego Instruments, San Diego, CA, USA). The apparatus consisted of a non-restrictive Plexiglas cylinder (inner diameter 8.8 cm, length 22.2 cm), mounted on a Plexiglas platform and placed in a ventilated, sound-attenuated chamber. A piezoelectric element detected cylinder movements. Background white noise (intensity 63 dB) was used to minimize the impact of external acoustic stimuli.

Animals were habituated to the apparatus (5 min daily) for 2 days before testing. In the trial day, following a 5-min acclimatization to the chamber, rats were presented five baseline startle stimuli (50 ms pulse of white noise at 120 dB) at a 30 s inter-stimulus interval, in order to become familiarized with the startle stimuli. Then, 60 startle stimuli were randomly presented (50 ms duration and variable intensity between 70 and 120 dB at 10 dB increments). Startle magnitude was assessed at 1 ms intervals, during the 200 ms period following stimulus. Chambers were cleaned between tests (70% ethanol and water) in order to remove olfactory cues.

Prepulse inhibition

The test lasted 20 min, following a 5 min acclimatization period. A white background noise (70 dB) was provided. Following five introductory 120-dB startle trials (noise lasting 40 ms), a total of 35 test trials were pseudo-randomly delivered as follows: (a) five trials with background noise only, (b) 10 startle trials of 120 dB and (c) five prepulses of each of four different intensities preceding a startle trial.

Prepulse intensities of 2, 4, 8, and 16 dB above the background noise level lasted 20 ms and preceded the 120 dB startle presentation in 100 ms. Intertrial intervals ranged from 10 to 20 s. The average startle response (AVG) was assessed in the 100 ms period following the onset of the startle stimulus presentation. The mean AVG for the ten 120 dB startle trials was employed in the formula used to assess the percentage of reduction in AVG compared to startle trials alone: % PPI=100 X [1−(AVG at prepulse plus startle trial)/(AVG at startle trial)].

Fear-potentiated startle

A stainless steel grid was adapted to the floor of the testing chamber, through which an electric current could be passed under software control. Following a 5-min acclimatization period, 20 light-shock pairings were presented at 30 s intervals. The shock (0.6 mA) was presented during the last 500 ms of the 5 s light pulse (3 W incandescent light bulb). Following conditioning, animals were returned to their home cages.

On the testing day, the steel grid used during conditioning, was maintained. After a 5-min acclimatization, 20 baseline startle stimuli were presented. Startles were measured. Then, animals were randomly presented 10 startle stimuli (intensity 120 dB, duration 50 ms), five of them during the last 50 ms of the delivery of a 5 s luminous conditioned stimulus (CS).

Histological procedures

Following behavioral assessment, eight male animals (derived from four to five different litters) of each group were placed under deep pentobarbital anesthesia and transcardially perfused with either 4% paraformaldehyde solution (N=4) or 0.9% saline (N=4).

Brains from the former set of subjects were embedded in glycolmethacrylate (Tecnovit 7100; Heraeus Kulzer, Werheim, Germany) and 30 μm coronal sections were obtained by microtome (Cerqueira et al. 2005). These were placed on a gelatinized slide, stained with Giemsa, mounted with Entellan (Merck, Darmstadt, Germany) and coverslipped. Shrinkage factor was calculated according to previous studies (Madeira et al. 1990).

Brains from animals perfused with 0.9% saline were processed for Golgi–Cox staining as previously described (Gibb and Kolb 1998); details are given in Electronic supplementary material (ESM).

Regional boundaries

We analyzed stereological parameters in three regions of the amygdaloid complex, namely the basolateral anterior (BLa), lateral (La) and central (CeA), as previously outlined (Paxinos and Watson 2005). CeA and La nuclei were differentiated using prior definitions (De Omlos et al. 2004) and the BLa division was distinguished from surrounding areas on the basis of cell size and staining intensity (De Omlos et al. 2004; Krettek and Price 1978). In accordance with a recent revision of anatomical and projection studies of the BNST (Dong et al. 2001), three divisions were considered, the anteromedial (BNSTam), anterolateral (BNSTal), and posterior (BNSTp). In order to avoid observer bias and to permit comparison between groups, the same observer drew all regional boundaries.

Stereological procedures

Volume and cell number estimations were obtained using StereoInvestigator® software (MicroBrightField, Williston, VT, USA) and a motorized microscope (Axioplan 2, Carl Zeiss, Hamburg, Germany) attached to a camera (DXC-390, Sony Corporation, Tokyo, Japan). The Cavalieri's principle (Gundersen et al. 1988) was applied to evaluate the volume of each region. Average cell numbers were estimated using the optical fractionator method (West et al. 1991). Coefficients of error were computed according to previously published formulas for cell numbers (Gundersen et al. 1999) and volume estimates (Gundersen and Jensen 1987). Detailed methods are provided in the ESM.

Dendritic tree analysis

BLa pyramidal-like, CeA multipolar, and BNSTam bipolar neurons were chosen as described elsewhere (McDonald 1982a, b, 1983). For each selected neuron, all branches of the dendritic tree were reconstructed at 600x magnification using a motorized microscope (Axioplan 2, Carl Zeiss), with oil-immersion objectives, and attached to a camera (DXC-390, Sony Corporation) and Neurolucida software (MicroBrightField). A 3-D analysis of the reconstructed neurons was performed using NeuroExplorer software (MicroBrightField). Dendritic branches were sampled in order to estimate spine density; spines in the selected segments were classified in thin, wide, ramified and mushroom categories (Harris et al. 1992). Thin spines were considered immature, while the other spine types were classified as mature. A total of 20 neurons/group/area were drawn (total 120 neurons).

Brain catecholamines

A different set of males was sacrificed by decapitation (n=6 animals derived from three different litters per group). After brain snap freezing, macrodissection of the amygdala and bed nucleus of stria terminalis was rapidly performed under a stereomicroscope (Model SZX7, Olympus America Inc.,

Center Valley, PA, USA). Whole brains were placed upside down and, using delicate forceps (Dumont #7 forceps, Fine Science Tools USA Inc., Foster City, CA, USA), the areas of interest were dissected according to stereological coordinates (Paxinos and Watson 2005). Samples were frozen in liquid nitrogen (overnight at −20°C) after adding perchloric acid 0.2 M; then, samples were briefly sonicated, centrifuged and 50 μl aliquots of the supernatant injected on a high performance liquid chromatography (HPLC) combined with electrochemical detection system. A mobile phase of 0.7 M aqueous potassium phosphate (monobasic; pH 3.0) in 10% methanol, 1-heptanesulfonic acid (222 mg/l), and Na-EDTA (40 mg/l) was used.

Levels of dopamine, 3,4-dihydroxyphenylacetic acid and 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid) were determined using a Gilson instrument (Gilson Inc., Middleton, WI, USA), fitted with an analytical column (Supelco Supelcosil LC-18 3 M; 7.5 cm×4.6 mm; flow rate: 1.0–1.5 ml/min; Supelco, Bellefonte, PA, USA). A standard curve was then obtained and data presented as concentration (nanograms per milligram of tissue protein).

Molecular correlates

In order to establish molecular correlates, the mRNA expression of dopamine D1 and D2 receptors (Drd1 and Drd2, respectively) was assessed in the BNST and amygdala. Moreover, we evaluated the expression of several synaptic plasticity-related genes, including synapsin (Syn), brain-derived neurotrophic factor (BDNF) and neural cell adhesion molecule (NCAM). The amygdala and bed nucleus of stria terminalis of an additional set of animals (N =8 animals derived from four different litters/group) were isolated as described above. For real-time PCR analysis, total RNA was isolated from frozen areas using Trizol (Invitrogen) and DNase treated (Fermentas), according to manufacturer. Two micrograms of RNA were converted into cDNA using the iSCRIPT kit (Biorad). RT-PCR was performed using SyberGreen (Qiagen) and the Biorad q-PCR CFX96 apparatus. HPRT was used as a housekeeping gene. We used relative quantification to determine the fold change difference between control and DEX animals, using the ΔΔCT method as described before (Pfaffl 2001). Primer sequences available in the ESM.

Statistical analysis

Results are presented as mean ± SEM. A repeated measures test was used to analyze data from acoustic startle, prepulse inhibition and fear-potentiated startle; Greenhouse-Geissser and Huynh–Feldt's corrections were applied for acoustic and fear-potentiated startle data, respectively. The comparison of means between groups was performed using the Student's t test for the remaining variables. Statistical significance was considered for $P<0.05$.

Results

Brief antenatal exposure to DEX triggers hyperanxiety and impairs fear conditioning

Animals exposed to either DEX 1 mg/kg or vehicle (controls) at embryonic day 18 and 19 were tested at 3 months of age. Antenatal exposure to DEX resulted in a significant reduction on the ratio of time spent in open arm when compared to controls (t=3.636, P=0.004; Fig. 1a); these results confirm our previous findings (Oliveira et al. 2006). Furthermore, while the number of open arm entries was also decreased following DEX exposure (t=2.424, P=0.026; Fig. 1b), there were no significant differences between groups in the number of closed arm entries (t=0.340, P=0.738), thus showing that the increased anxiety behavior should not be attributed to locomotory differences.

In the acoustic startle test, startle amplitudes for DEX-exposed subjects increased more rapidly as a function of stimulus intensity when compared to controls ($F_{(3,47)}$= 4.036, P=0.016; Table 1). When comparing responsiveness to individual noise intensities between groups, DEX-exposed rats displayed significantly increased startle amplitudes at 70 (t=5.121, $P<0.001$), 80 (t=4.252, P=0.001), 90

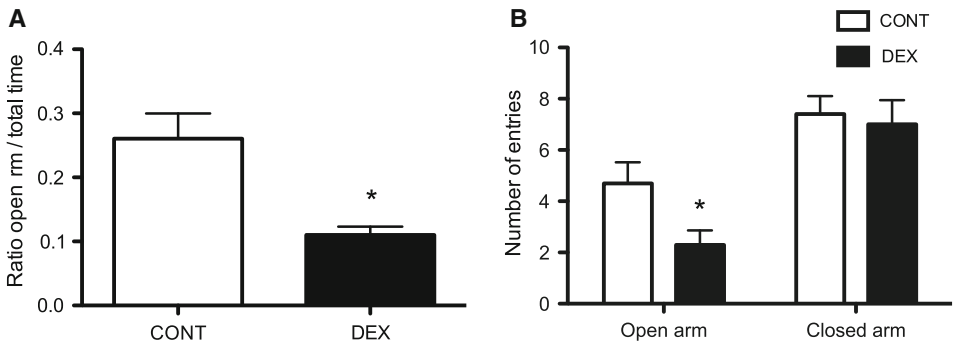

Fig. 1 Elevated plus maze data. **a** Ratio of time spent in open arm over total time. **b** Number of open and closed arm entries. Data presented as mean ± SEM. *Different from controls, $P<0.05$

Table 1 Acoustic startle data

Noise (dB)	Controls	DEX
70 dB	30.51±3.03	97.50±15.07 *
80 dB	32.50±4.72	291.17±86.56 *
90 dB	145.19±21.39	561.26±115.66 *
100 dB	1203.54±168.88	3126.46±608.44 *
110 dB	4645.30±461.93	6221.83±447.09 *
120 dB	6358.69±257.17	7101.62±519.47

Startle amplitude (arbitrary units) in response to acoustic stimulus. Data presented as mean ± SEM

*$P<0.05$, different from controls

Table 2 Fear-potentiated acoustic startle data

	Controls	DEX
Startle	5290.89±658.66	5252.08±716.75
CS+startle	6229.04±425.83	4554.13±577.61*

Startle amplitude (arbitrary units) in response to acoustic stimulus with or without previous conditioned stimulus (CS). Data presented as mean ± SEM

*$P<0.05$, different from controls

(t=3.537, P=0.006), 100 (t=3.045, P=0.012) and 110 dB (t=2.452, P=0.025).

Interestingly, the acoustic startle response to inhibitory prepulses was not affected by exposure to antenatal DEX, as shown by the analysis of treatment×prepulse intensity interaction ($F_{(3,54)}$=3.858, P=0.994; Fig. 2). Moreover, comparison between groups failed to reveal significant differences at any prepulse intensity. The fact that all groups had similar percentages of reduction in the average of startle response, supports the absence of sensorimotor deficits.

Startle amplitude varied as a function of the treatment× stimulus interaction in the fear-potentiated startle ($F_{(1,18)}$= 8.379; P=0.01; Table 2). While controls displayed enhanced startle amplitude after being presented a conditioned stimulus startle, DEX exposure resulted in an impaired fear conditioning in the acoustic startle, as shown by the significantly decreased ratio of conditioned stimulus/basal startle responsiveness in the DEX group (t=−2.602, P=0.018; Fig. 3).

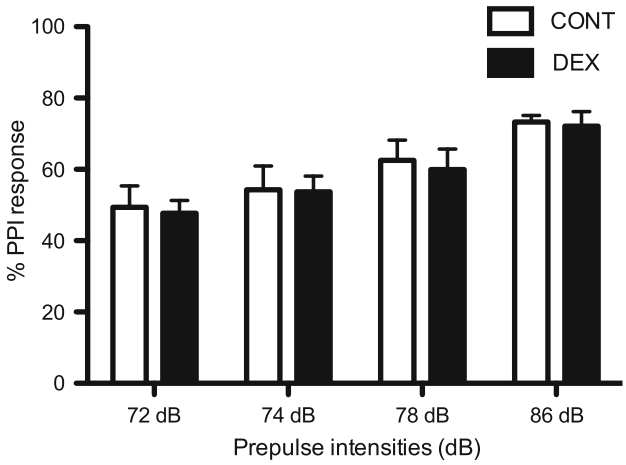

Fig. 2 Prepulse inhibition test. Percentages of reduction in average startle response compared with startle trials at prepulse intensities of 2, 4, 8, and 16 dB above background noise level

Antenatal DEX exposure promotes dendritic remodeling in the BNST and in the amygdala

To further understand the behavioral findings, we have performed a detailed morphological characterization of the BNST and amygdala brain regions, known to be involved in fear and anxiety, respectively (Walker and Davis 1997).

Volumetric determinations reveal that antenatal exposure to DEX resulted in an increase in BNST total volume (t= 3.841, P=0.009; data not shown). This effect was largely due to increased volumes in anteromedial division of the BNST of DEX-exposed animals (t=5.887, P=0.001; Fig. 4a); conversely, the volumes of BNSTal and BNSTp were not influenced by such antenatal exposure (t=−0.484, P=0.646 and t=0.718, P=0.500, respectively). DEX exposure during pregnancy did not affect the total number of cells in the BNST (t=1.595, P=0.162) nor in its divisions (BNSTam: t=1.475, P=0.191; BNSTal: t= −1.506, P=0.183; BNSTpost: t=0.946, P=0.381; Fig. 4b).

The volumetric increase in the BNST was further scrutinized through a 3-D morphological analysis of dendritic arborizations of neurons in the BNSTam; the neurons in this brain region were previously described as cells with ovoid soma, moderate polarized dendritic

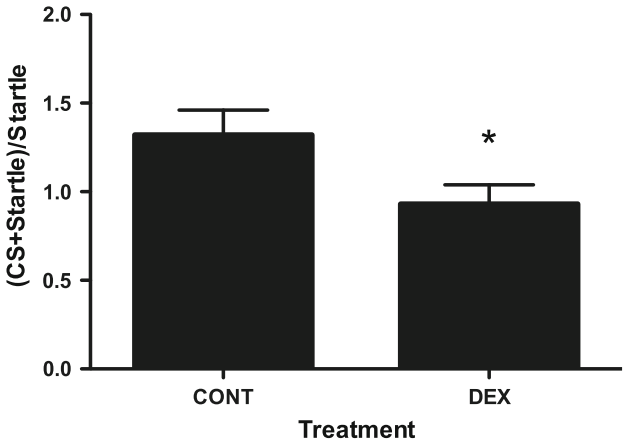

Fig. 3 Fear-potentiated acoustic startle data. Data presented as the average of ratios between conditioned stimulus/basal startle responsiveness ± SEM. *Different from controls, $P<0.05$

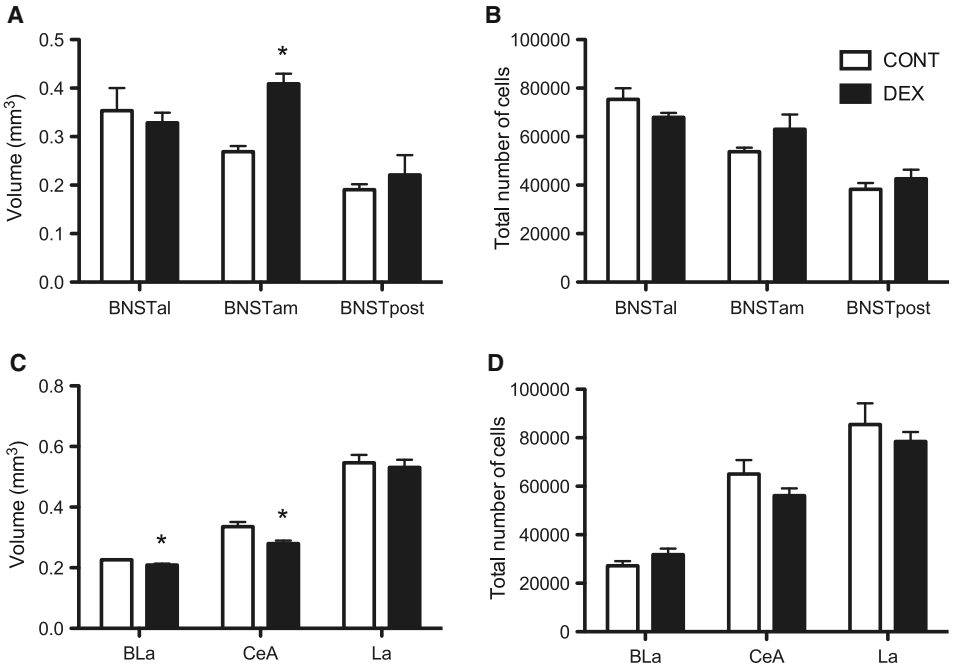

Fig. 4 **a** Estimated volumes of anterolateral (BNSTal), anteromedial (BNSTam) and posterior (BNSTpost) divisions of the bed nucleus of stria terminalis. Data presented in cubic millimeters, as mean ± SEM. **b** Estimated total number of cells in the anterolateral (BNSTal), anteromedial (BNSTam) and posterior (BNSTpost) divisions of the bed nucleus of stria terminalis. **c** Estimated volumes of BLa, CeA, and La divisions of the amygdala. Data presented in cubic millimeters, as mean ± SEM. **d** Estimated total number of cells in the BLa, CeA, and La divisions of the amygdala. Data presented as mean ± SEM; *Different from controls, $P<0.05$

branching, with sparse spines (McDonald 1983). Data revealed that antenatal exposure to DEX resulted in a significant increase of total dendritic length when compared to controls ($t=4.498$, $P<0.001$; Table 3). Spine densities were not affected by antenatal DEX treatment ($t=-1.779$, $P=0.085$), even when a separate analysis of mature ($t=-1.825$, $P=0.076$) and immature forms ($t=-0.534$, $P=0.0596$) was performed.

Table 3 Dendritic length and spine density of bipolar neurons of anteromedial region of the bed nucleus of stria terminalis (BNSTam), pyramidal-like neurons of basolateral amygdaloid nucleus (BLa), and multipolar neurons of the central nucleus of the amygdala (CeA)

	Controls	DEX
BNSTam (bipolar)		
Dendritic length (μm)	395±17	511±19*
Density of spines (n/μm)	0.58±0.042	0.49±0.027
Mature	0.47±0.035	0.39±0.026
Immature	0.10±0.015	0.09±0.016
BLa (pyramidal)		
Dendritic length (μm)	1969±128	1506±137*
Basal dendrites	1088±87	721±72*
Apical dendrite	881±80	785±83
Density of spines (n/μm)	0.95±0.046	0.95±0.027
Mature spines	0.71±0.037	0.70±0.026
Immature spines	0.24±0.022	0.26±0.012
CeA		
Dendritic length (μm)	818±38	670±19*
Density of spines (n/μm)	0.45±0.043	0.47±0.037
Mature	0.35±0.039	0.36±0.032
Immature	0.09±0.009	0.12±0.015

Data presented as mean ± SEM

*$P<0.05$, different from controls

In contrast to the BNST, antenatal exposure to DEX resulted in a decreased volume of BLa and CeA divisions of the amygdala (BLa $t=-3.564$, $P=0.02$; CeA $t=-3.072$, $P=0.027$) but not in the La division ($t=-0.561$, $P=0.605$) (Fig. 4c). No effect was observed on the estimated number of cells in the BLa, CeA and La divisions of the amygdala ($t=1.457$, $P=0.196$; $t=-1.383$, $P=0.216$; $t=-0.725$, $P=0.496$, respectively; Fig. 4d).

The analysis of the dendritic arborizations of pyramidal-like neurons in the BLa region of the amygdala, which represent the predominant cell type in the area (70%; type I) (McDonald 1982b; Sah et al. 2003), revealed a reduction in total and basal dendritic lengths following DEX treatment ($t=-2.471$, $P=0.018$ and $t=-3.252$, $P=0.002$, respectively; Table 3); however, apical dendritic length was not affected ($t=-0.832$, $P=0.411$) by antenatal DEX exposure. The density of spines in these neurons was not affected by antenatal exposure to DEX ($t=0.165$, $P=0.870$), even when assessing mature and immature spines separately ($t=-0.122$, $P=0.903$ and $t=0.598$, $P=0.555$, respectively).

Treatment also affected the CeA predominant cell type, previously called medium spiny neurons (by comparison with neurons in the striatum; Sah et al. 2003). These neurons have an ovoid or fusiform soma and three to five nonspiny primary dendrites from which moderately spiny, sparsely branching secondary and tertiary dendrites arise. These neurons in the DEX progeny displayed a significant

dendritic atrophy when compared to controls (t=−3,491, P=0.002; Table 3). No effect was found on the densities of total, mature and immature spines (t=0.435, P=0.666, t=0.023, P=0.982 and t=1.332, P=0.191, respectively).

Neurochemical and molecular correlates

To complement the behavioral and morphometric analysis, we decided to measure dopamine levels and its metabolites. Antenatal DEX exposure had no effect on the concentration of dopamine in the BNST, nor its turnover (dopamine t=0.026, P=0.980; dopamine turnover t=0.291, P=0.777; Fig. 5a–b).

In contrast, the neurochemical analysis in the amygdala revealed a decrease in dopamine concentration following antenatal exposure to DEX (t=5.006, P=0.001; Fig. 5c). Moreover, dopamine turnover was significantly increased in these subjects (t=−4.405, P=0.001; Fig. 5d).

At a molecular level, and given the impact of antenatal DEX exposure on dendritic arborization, we assessed the expression of the synaptic plasticity-related genes in the BNST and amygdala, namely a synaptic gene (synapsin (Syn)), a neurotrophin (brain-derived neurotrophic factor (BDNF)) and a cell adhesion molecule (neural cell adhesion molecule (NCAM)), all known to be involved in synaptic/dendritic plasticity. Moreover, as the dopaminergic innervation of the BLa is considered to facilitate amygdala-dependent functions (Asan 1997), and since a hypodopaminergic status was previously associated with DEX exposure (Leao et al. 2007; Rodrigues et al. 2010), the expression of dopamine D1 and D2 receptors (Drd1 and Drd2, respectively) was also evaluated.

In the BNST, DEX-exposed animals displayed increased expression of synapsin (t=−2.418, P=0.030) and NCAM (t=−2.032, P=0.045) (Table 4). No significant differences between groups were found in the expression of the remaining genes analyzed (Drd1 t=−0.663, P=0.514; Drd2 t=−0.881, P=0.386; BDNF t=−0.214, P=0.832).

In the amygdala, Drd2 mRNA levels were significantly increased in the DEX-exposed subjects (t=−3392, P=0.008), while Drd1 were not affected (t=0.370, P=0.717; Table 4). The expression levels of the synaptic plasticity-related genes, namely synapsin, BDNF and NCAM were not significantly different between groups (t=0.546, P=0.594, t=−1.457, P=0.167 and t=−0.376, P=0.713, respectively).

Discussion

In utero and early postnatal environment, namely the GC milieu, is crucial for neurodevelopment not only through its activational effects, but also through important programming effects. Increasing evidence suggests that early life exposure to GC triggers undesired metabolic, cardiovascular, neuroendocrine and behavioral phenotypes in adulthood (Mesquita et al. 2009). Both synthetic (such as DEX) and naturally occurring GCs are able to exert a programming effect in the brain; however, it is important to remember that their effects can be quite distinct (Oliveira et al. 2006) as endogenous GCs preferentially bind to MR, whereas

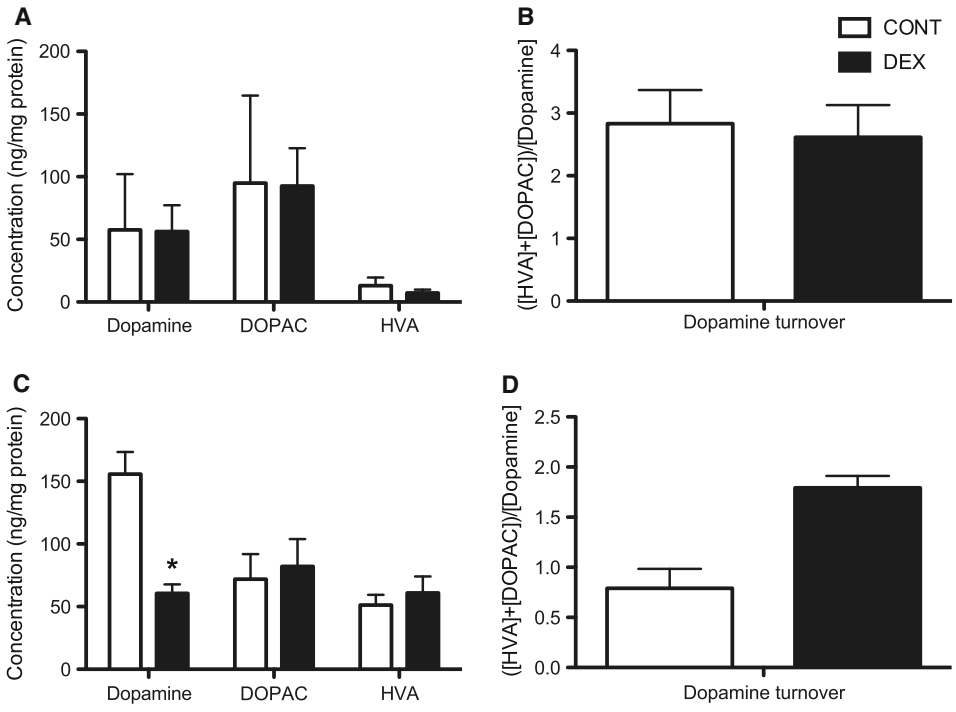

Fig. 5 Neurochemical analysis by HPLC of the bed nucleus of stria terminalis (BNST) (**a**–**b**) and the amygdala (**c**–**d**). **a**, **c** Concentration of each neurotransmitter (nanograms per milligram of protein) is presented. 3,4-dihydroxyphenylacetic acid (DOPAC) and 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid, HVA). **b**, **d** Dopamine turnover assessed by the ratio between dopamine metabolites and dopamine concentrations. Data presented as mean ± SEM

Table 4 Gene expression analysis (measured by real-time PCR) in amygdala and bed nucleus of stria terminalis (BNST)

Treatment	Drd1	Drd2	Syn	BDNF	NCAM
BNST					
Controls	0.950±0.12	1.070±0.12	0.918±0.05	1.127±0.14	0.987±0.04
DEX	1.229±0.21	1.226±0.13	1.200±0.11*	1.196±0.27	1.318±0.25*
Amygdala					
Controls	1.066±0.13	0.927±0.07	1.167±0.19	0.920±0.04	1.043±0.10
DEX	0.990±0.16	2.494±0.62*	1.060±0.04	1.102±0.12	1.115±0.17

The mRNA expression levels are presented as the fold change increase in relation to the respective control. Data presented as mean ± SEM. Dopamine D1 receptor (Drd1), dopamine D2 receptor (Drd2), synapsin (Syn), brain-derived neurotrophic factor (BDNF) and neural cell adhesion molecule (NCAM)

*$P<0.05$, different from controls

DEX binds almost exclusive to GR (Reul et al. 1987), the receptors that mediate most of the detrimental effects of GCs. Although it has been argued that synthetic GCs have a limited capacity to reach the brain in mice (De Kloet et al. 1975), its deleterious effects in the central nervous system are undeniable (Cerqueira et al. 2005; Cerqueira et al. 2007; Leao et al. 2007; McArthur et al. 2005; Yu et al. 2010), specially in rats, and may arise from the use of higher dosages which can indeed pass the barriers and activate brain GC receptors (Miller et al. 1992; Reul et al. 1987). Moreover, in the context of the present study, it is important to highlight the fact that the exposure to DEX has occurred during a period in which the brain barriers are still immature (Saunders et al. 2000).

The data herein presented extends our previous observations that antenatal exposure to DEX results in anxiety-like behavior in adult animals (Oliveira et al. 2006). In fact, while we confirmed a hyperanxious phenotype in the elevated plus maze, we also found an increased acoustic startle response (ASR) in DEX-exposed rats. The startle, a fast protective response from the organism to a sudden and intense stimulus (Koch 1999), is considered an indicator of anxiety-like behavior. The ASR results from the activation of a trisynaptic circuit, where auditory inputs from several brainstem nuclei (dorsal cochlear nucleus, cochlear root nucleus, ventral cochlear nucleus, lateral superior olive and ventrolateral tegmental nucleus) are conveyed to a main sensorimotor interface, the caudal pontine reticular nucleus, which then projects to spinal motor neurons to trigger the motor response (Koch 1999; Lee et al. 1996). Importantly, it was demonstrated that a direct projection from the BNST to the caudal pontine reticular nucleus, but also an indirect one via the CeA, are responsible for the modulatory response of stressors upon the ASR (Davis et al. 1997).

The BNST is one of the relay stations that conveys inputs from stress-sensitive areas of the cortex and limbic system both to the HPA axis but also to brain stem nuclei implicated in several emotional behaviors (Herman and Cullinan 1997). Here, we show a hypertrophy of the BNST, due to the enlargement of its anteromedial region. Interestingly, this region presented alterations in the expression levels of NCAM, a molecule important for neuronal plasticity (Bisaz and Sandi 2010; Nacher et al. 2002). Augmented levels of synapsin, a protein involved in the regulation of neurotransmitter release (Rosahl et al. 1995), also suggest increased synaptic activity of this brain region in DEX-exposed animals, which is in line with the increased dendritic arborization of these animals. Such plastic changes are paralleled with an increased startle response, thus supporting the idea of an overactivation of this brain subregion. This increased activity of the BNSTam is also in accordance with our previous observation of a hyperresponsive HPA axis in these subjects (Oliveira et al. 2006). Importantly, the activational increase of circulating corticosteroids might further promote the activation of the BNST as it is known that these hormones are implicated in the neuroanatomical changes observed in this brain region (Pego et al. 2010).

Several projections to the caudal pontine reticular nucleus are considered responsible for the increase in startle amplitude induced by fear conditioning to a previous neutral stimulus. These include projections from the CeA, either direct or via mesencephalic reticular formation and deep layers of superior colliculus, but also indirect projections from the medial amygdala via ventromedial hypothalamus and ventral periaqueductal gray (Davis 2006). Previous data supports that while baseline startle amplitudes are not affected by interference with these connections, the response to fear conditioning is. Interestingly, we here show that antenatal DEX exposure impairs fear conditioning in adulthood, which is consistent with deficits in amygdalar function and memory consolidation for emotionally arousing experiences. These results, together with previous data showing that early life stress (neonatal isolation) resulted in impairment of context-induced fear conditioning in adult male rats (Kosten et al. 2006),

confirms that fear conditioning can be modulated by adverse early life events. The role of different divisions of the amygdala in fear behavior, in particular the CeA and BLa, has been extensively studied. Several forms of CeA lesions have been correlated with disruption of fear-potentiated startle (Campeau and Davis 1995; Hitchcock and Davis 1987; Walker and Davis 1997). Electrolytic lesions of the CeA completely blocked the expression of fear-potentiated startle in rats (Kim and Davis 1993). Conversely, inactivation of BNST did not disrupt fear-potentiated startle (Gewirtz et al. 1998; Walker and Davis 1997); however, more recently, it has been suggested that BNSTlat tonically inhibits fear-potentiated startle, probably through projections to the CeA (Meloni et al. 2006). Thus, the present observations of decreased volumes in the CeA and BLa in DEX-exposed rats are likely to be implicated in the changes in fear conditioning displayed by these animals; once again, the volumetric decreases in these amygdalar divisions result largely from dendritic atrophy—which fits previous observations of the effects of chronic unpredictable stress in the BLa (but not the CeA; Vyas et al. 2003; Vyas et al. 2002). It is relevant to stress at this point that others have shown that a single course of betamethasone at postnatal day one failed to affect volumes of cerebral cortex, corpus callosum, hippocampus, dentate gyrus or amygdala (Yossuck et al. 2006), however, several technical differences may explain this discrepancy. The time of the exposure and the nature of insults seems to be critical issues to consider, as several windows of vulnerability to the programming effects of different stimuli seem to occur. Indeed, stress effects in neuronal morphology of specific brain regions are not necessarily equal across life and depend on the type of stress. While stress effects in the BNST morphology seem more similar, since different types of stress induce a general hypertrophy of dendrites in the neurons of this brain region (Pego et al. 2008; Vyas et al. 2003), the effects of stress in the amygdala are less concordant to our present observation of dendritic atrophy. Whereas chronic juvenile stress leads to a general hypertrophy of amygdalar dendrites (Eiland et al. 2011), others failed to find any significant change in dendritic structure after stress exposure in adulthood (Pego et al. 2008) and others have shown remarkably divergent changes in amygdalar neurons following different types of stress (Vyas et al. 2003; Vyas et al. 2002).

Remarkably, such morphological effects in the amygdala were accompanied by changes in dopamine but not other catecholamines levels. Dopamine, arising mainly from the VTA, plays a facilitative role in the function of the amygdala (Asan 1997). In vitro studies also suggest that the excitability of amygdalar, namely BLa, neurons is modulated by dopamine (Kroner et al. 2005). In addition, behavioral data showed that VTA lesion results in blocked fear-potentiated startle (Borowski and Kokkinidis 1996). At least in part, the facilitation of affective behaviors by DA may be explained by actions at the cellular level on BLa neurons. It has been suggested that DA receptor activation could simultaneously facilitate the BLa output in response to strong inputs that cause spike firing, while suppressing weaker inputs via activation of GABAergic interneurons (Kroner et al. 2005). Thus, the present observation of a hypodopaminergic status in the amygdala seems to be of relevance to the deficits in fear memory. In fact, it confirms observations in models of dopamine deficient mice (Fadok et al. 2010), in which the restoration of dopamine in the BLa was shown to be required for the formation of fear-related memory (Fadok et al. 2010). Dopamine levels in the amygdala are also determinant for sensorimotor gating, as it was shown that NAcc and amygdalar infusions of this neurotransmitter significantly impair PPI (Swerdlow et al. 1992); surprisingly, we did not find any differences in PPI in DEX-exposed animals, which is also in accordance with another previous study (Hauser et al. 2006).

Herein, the decreased amygdalar levels of dopamine are associated with increased expression of D2 receptor mRNA in DEX-exposed subjects; such up-regulation could be seen as a compensation mechanism. Remarkably, no differences were found in D1 receptor expression, suggesting that D2 receptor has a prominent role in fear behavior. Indeed, while D1 receptors may participate in recognition of danger, D2 receptors seem to have a role in setting up adaptive responses to adverse stimuli (de la Mora et al. 2010). Moreover, D2 (but not D1) antagonists injection in the BLa impairs fear-potentiated startle probably due to reduced dopaminergic tone (de Oliveira et al. 2011), which is in agreement with the amygdalar hypodopaminergic status seen in DEX-exposed animals. These findings are consistent with recent data showing that D2 receptor pathway connecting the VTA and BLa modulates conditioned fear (de Oliveira et al. 2011).

The present findings unravel the impact of antenatal exposure to DEX in the brain regions implicated in fear and anxiety behaviors. We show that subjects exposed to GCs during neurodevelopment present marked neuroanatomical, neurochemical and molecular programming changes in the BNST and amygdala, and are more vulnerable to anxiety and fear pathology in adulthood. Thus, our findings further support the concerns raised on the potential deleterious effects of antenatal exposure to synthetic corticosteroids (Rodrigues et al. 2010, 2011; Talge et al. 2007; Mesquita et al. 2009) and call for the need of a parsimonious use of these drugs.

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Disclosure/conflict of interest The authors have no conflicts of interest to declare.

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SUPPLEMENTARY MATERIAL

Histological procedures

Following behavioral assessment, animals were placed under deep pentobarbital anesthesia and transcardially perfused with either 4% paraformaldehyde solution (N=4/group) or 0.9% saline (N=4/group).

Brains from the former set of subjects were embedded in glycolmethacrylate (Tecnovit 7100; Heraeus Kulzer, Werheim, Germany) and 30 μm coronal sections were obtained by microtome (Cerqueira et al., 2005). These were placed on a gelatinized slide, stained with Giemsa, mounted with Entellan (Merck, Darmstadt, Germany) and coverslipped. Shrinkage factor was calculated according to previous studies (Madeira et al., 1990).

Brains from animals perfused with 0.9% saline were processed for Golgi-Cox staining as previously described (Gibb and Kolb, 1998). In brief, brains were removed and immersed in Golgi-Cox solution [1:1 solution of 5% potassium dichromate and 5% mercuric chloride diluted 4:10 with 5% potassium chromate (Glaser and Van der Loos, 1981)] for 14 days; brains were then transferred to a 30% sucrose solution (7 days), before being cut on a vibratome. Coronal sections (200 μm thick) were collected in 6% sucrose and blotted dry onto cleaned, gelatin-coated microscope slides. They were then alkalinized in 18.7% ammonia, developed in Dektol (Kodak, Linda-a-Velha, Portugal), fixed in Kodak Rapid Fix (prepared to manufacturer's instructions), dehydrated through a graded series of ethanols, and cleared in xylene prior to being mounted and coverslipped.

Stereological procedures

Volume and cell number estimations were obtained using StereoInvestigator® software (MicroBrightField, Williston, VT, USA) and a motorized microscope (Axioplan 2, Carl Zeiss, Hamburg, Germany) attached to a camera (DXC-390, Sony Corporation, Tokyo, Japan). The Cavalieri´s principle (Gundersen et al., 1988) was applied to evaluate the volume of each region. Every fourth section was used and its cross-sectional area was estimated by point counting at a final magnification of 112x. A test-point system with an interpoint distance at

the tissue level of 120 µm, was used. The volume of the region of interest was then calculated from the number of points that fell within its boundaries and the distance between the systematically sampled sections.

Average cell numbers were estimated using the optical fractionator method (West et al., 1991). In brief, a grid of equally spaced virtual 3-D boxes (30 µm x 30 µm x 20 µm) was superimposed on every fourth section, as for volumetric estimation. The number of cells falling inside the boxes was counted, according to standardized stereological procedures (Gundersen et al., 1999; Coulin et al., 2001). In order to exclude glial cells from estimates, neuronal and glial cell body profiles were considered, based on criteria described elsewhere (Ling et al., 1973; Peinado et al., 1997). Coefficients of error were computed according to previously published formulas for cell numbers (Gundersen et al., 1999) and volume estimates (Gundersen and Jensen, 1987).

Dendritic tree analysis

BLa pyramidal-like, CeA multipolar and BNSTam bipolar neurons were chosen as described elsewhere (McDonald, 1982b, a, 1983). For each selected neuron, all branches of the dendritic tree were reconstructed at 600x magnification using a motorized microscope (Axioplan 2, Carl Zeiss), with oil-immersion objectives, and attached to a camera (DXC-390, Sony Corporation) and Neurolucida software (MicroBrightField). A 3-D analysis of the reconstructed neurons was performed using NeuroExplorer software (MicroBrightField). The criteria used to select neurons for reconstruction were: a) full impregnation of the neurons along the entire length of the dendritic tree; b) dendrites without significant truncated branches; c) no morphological changes attributable to incomplete dendritic impregnation of Golgi-Cox staining; d) relative separation from neighboring impregnated neurons. Dendritic branches were sampled in order to estimate spine density (number of spines per micron of dendritic length), using the following method: only branches that were either parallel or at acute angles to the coronal surface of the section and did not show overlap with other branches that would obscure visualization of spines were considered; moreover, segments were randomly selected in the proximal parts of the tree; spines in the selected segments were classified in thin, wide, ramified and mushroom categories (Harris et al., 1992). Thin

spines were considered immature, while the other spine types were classified as mature. A total of 20 neurons/group/area were drawn (total 120 neurons).

Molecular correlates

Primer Name	Sequence
Hprt_F	GCAGACTTTGCTTTCCTTGG
Hprt_R	TCCACTTTCGCTGATGACAC
NCAM_F	AAAGGATGGGGAACCCATAG
NCAM_R	TAGGTGATTTTGGGCTTTGC
Synapsin_F	CACCGACTGGGCAAAATACT
Synapsin_R	TCCGAAGAACTTCCATGTCC
Bdnf_F	GCGGCAGATAAAAAGACTGC
Bdnf_R	GCAGCCTTCCTTCGTGTAAC
Drd1_F	TCCTTCAAGAGGGAGACGAA
Drd1_R	CCACACAAACACATCGAAGG
Drd2_F	CATTGTCTGGGTCCTGTCCT
Drd2_R	GACCAGCAGAGTGACGATGA

Table 1 - Primer sequences for assessment of mRNA expression of neural cell adhesion molecule (NCAM), synapsin, brain-derived neurotrophic factor (BDNF) and dopamine D1 (Drd1) and D2 receptors (Drd2).

Chapter 2.3

Oliveira M, Leão P, Rodrigues AJ, Pêgo JM, Cerqueira JJ, Sousa N

Programming effects of antenatal corticosteroids exposure in male sexual behavior

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Programming Effects of Antenatal Corticosteroids Exposure in Male Sexual Behavior

Mário Oliveira, MD, Pedro Leão, MD, PhD, Ana-João Rodrigues, PhD, José-Miguel Pêgo, MD, PhD, João-José Cerqueira, MD, PhD, and Nuno Sousa, MD, PhD

Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga, Portugal

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ABSTRACT

Introduction. Brain regions implicated in sexual behavior begin to differentiate in the last trimester of gestation. Antenatal therapy with corticosteroids is often used in clinical practice during this period to accelerate lung maturation in preterm-risk pregnancies. Clinical and animal studies highlighted major behavioral impairments induced later in life by these treatments, especially when synthetic corticosteroids are used.

Aim. To evaluate the implications of acute prenatal treatment with natural vs. synthetic corticosteroids on adult male rat sexual behavior and its neurochemical correlates.

Methods. Twelve pregnant Wistar rats were injected with dexamethasone (DEX—1 mg/kg), corticosterone (CORT—25 mg/kg), or saline on late gestation (pregnancy days 18 and 19). Following this brief exposure to corticosteroids, we assessed the sexual behavior of the adult male progeny and subsequently associated these behaviors with the levels of catecholamines and mRNA of dopamine and androgen receptors (AR) in brain regions relevant for sexual behavior.

Main Outcome Measures. Sexual behavior of adult male offspring was assessed by exposure to receptive females. This was associated with serum testosterone levels and levels of catecholamines (determined by high-performance liquid chromatography) and dopamine and AR mRNA expression (real-time polymerase chain reaction [PCR]) in brain regions implicated in sexual behavior.

Results. Prenatal DEX exposure resulted in a decreased number and increased mounts and intromissions latencies in adulthood. These findings were associated with decreased levels of serum testosterone and increased hypothalamic expression of AR mRNA. DEX animals also displayed lower dopamine levels and higher dopamine receptor mRNA expression both in hypothalamus and nucleus accumbens (NAcc). The milder phenotype of CORT animals was associated only with decreased dopamine levels in NAcc.

Conclusion. Antenatal corticotherapy programs adult male sexual behavior through changes in specific neuronal and endocrine mediators. Importantly, equipotent doses of CORT trigger less detrimental consequences than DEX, emphasizing the differential impact of activation of the different corticosteroid receptors. **Oliveira M, Leão P, Rodrigues A-J, Pêgo J-M, Cerqueira J-J, and Sousa N. Programming effects of antenatal corticosteroids exposure in male sexual behavior. J Sex Med 2011;8:1965–1974.**

Key Words. Antenatal Corticotherapy; Corticosteroids; Dopamine; Neurodevelopment; Sexual Behavior; Central Neurochemichal Correlates

Introduction

The last trimester of gestation and early postnatal period are critical for brain sexual differentiation [1]. Insults at this period, including stress and prolonged exposure to corticosteroids, have been shown to disrupt several behaviors in adulthood, namely male sexual behavior [2–4]. Interestingly, exposure to corticosteroids during late gestation has been correlated with a sustained perturbation in male steroidogenesis [5] and an impoverished dopaminergic innervation of the nucleus accumbens (NAcc) [6], which is of particular relevance when considering the facilitatory role

of dopamine in the different aspects of sexual behavior [7]. In contrast to dopamine, serotonin is primarily inhibitory especially at the hypothalamus level [8–10]. In fact, it is the balance of serotonin and dopamine levels at specific areas that controls almost all aspects of sexual behavior [8].

Importantly, the levels of dopamine and serotonin can be modulated by testosterone [8]. Testosterone induces an up-regulation of nitric oxide synthase [11] and, concomitantly, dopamine release in preoptic area [12,13], which strongly influences male sexual behavior. Testosterone may also have a direct activational effect on androgen receptors (AR) and an indirect one (via estradiol formation) on estrogen receptors [14], important mediators of sexual behavior response.

In clinical practice, glucocorticoids are prescribed in about 10% of pregnancies at risk of preterm delivery in order to promote fetal lung maturation [15–17]. Dexamethasone (DEX) and betamethasone, the preferred drugs [18], are synthetic corticosteroids that cross the placenta with 100% efficacy and have been shown to reduce the morbidity and mortality of the preterm infant after delivery [17]. Despite this, the safety of the exposure of the developing fetal brain to glucocorticoids has been questioned as it might have lifelong effects on adult behavior and neuroendocrine function [19–21]. Available data suggest that the activity of the hypothalamic–pituitary–adrenal (HPA) axis, which is vital to stress response, might be reprogrammed by manipulations in the corticosteroid milieu during late gestation; this altered pattern of the HPA is believed to be, at least in part, responsible for the behavioral and neuroendocrine changes [22], as well as for increased risk for hypertension, type 2 diabetes [23–25], and neuropsychiatric disorders [21]. Of particular interest is the evidence suggesting a less deleterious effect on adult emotional behavior of the acute administration of endogenous corticosteroids [20], especially in light of evidence showing that cortisol displays similar therapeutic efficacy to DEX during pregnancy and neonatal life [16].

In light of this evidence, we decided to assess the impact of short-term antenatal corticosteroid exposure in male sexual behavior and search for its neurochemical and endocrine correlates. Furthermore, we also wanted to compare natural and synthetic corticosteroids in terms of long-term adverse effects.

Methods

Animals and Treatments

Experiments were conducted in accordance with local regulations (European Union Directive 86/609/EEC) and National Institutes of Health guidelines on animal care and experimentation.

Twelve adult pregnant Wistar Han rats (Charles-River Laboratories, Barcelona, Spain) received at day 14 of gestation were individually housed under standard laboratory conditions (12/12 hours light/dark cycle, with lights on at 8 AM; food and water ad libitum).

Subcutaneous injections of DEX (1 mg/kg, Sigma-Aldrich, St. Louis, MO, USA; N = 4), corticosterone (CORT, 25 mg/kg, Sigma-Aldrich; N = 4), or saline (controls, 1 mL/kg; N = 4) were administered on embryonic days (ED) 18 and 19 of pregnancy [20]. Drug dosages were chosen to achieve comparable transrepressive potencies at the glucocorticoid receptors [26].

Weaning was performed at postnatal day 21 and pups were pair-housed according to gender and prenatal experimental procedures. Male offsprings (two siblings per dam; N = 4 dams/group) were tested at 3 months for sexual behavior.

Preparation of Sexually Receptive Females

Adult female rats (3 months) were individually housed and ovariectomized, as previously described [27]. Sexual receptivity was induced by subcutaneous estradiol benzoate (20 μg/rat, Sigma-Aldrich) and progesterone (1 mg/rat, Sigma-Aldrich) 52 and 4 hours before male exposure, respectively.

Male Sexual Behavior

The test arena consisted of a rectangular Plexiglas box (40 × 60 × 40 cm) with a transparent top and a video camera over it. Males were exposed to females 2 hours after the onset of the dark phase, in a quiet room, with a dim red light. Sexually experienced males were placed in the arena 10 minutes before a receptive female was presented and activity was recorded for 20 minutes; mount and intromission latency and number of mounts and intromissions (vaginal penetration) were registered manually by the observer and intromission ratio calculated as intromissions/(intromissions + mounts). Numbers of ejaculations were assessed visually for each animal.

Biometric and Testosterone Measurements

Animals were sacrificed 1 week after behavior assessment and blood collected for determination

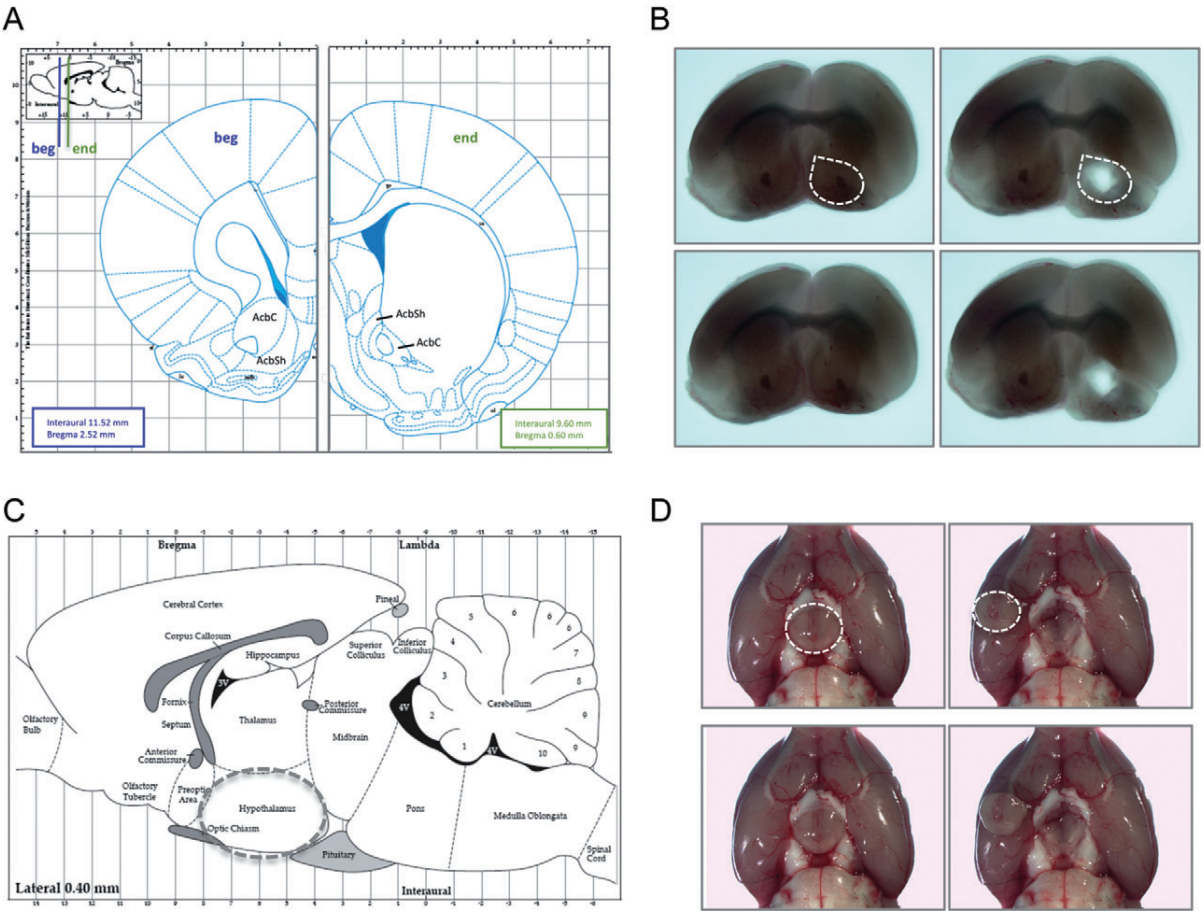

Figure 1 Macrodissection of the nucleus accumbens (NAcc) and hypothalamus. (A) Schematic representation of the beginning (beg) of the NAcc, at interaural 11.52 mm and bregma 2.52 mm and the end (end) of this area at interaural 9.60 and bregma 0.60. It is possible to distinguish the NAcc core (AcbC) and the shell (AcbSh). (B) Example of the NAcc dissection. Brains were cut using brain slicer following adequate references and whole NAcc was removed using delicate forceps. (C) Schematic representation of the rat brain with the hypothalamus highlighted. (D) Dissection of the hypothalamus. Schemes on A and B were adapted from Paxinos and Watson [28].

of serum total testosterone levels by electrochemiluminescense immunoassay (Elecsys Testosterone II reagent kit, Roche Diagnostics, Indianapolis, IN, USA; measuring range 2.5–1,500 ng/dL). Testis wet weight was assessed and normalized to the total weight of the animal.

Brain Catecholamines

After brain snap freezing, the regions of interest were rapidly dissected under the scope using macrodissection of specific brain areas (Figure 1). The hypothalamus was isolated by placing whole brains upside down and using delicate forceps (Dumont #7 forceps, Fine Science Tools USA Inc., Foster City, CA, USA) to detach it from the rest. The hypothalamus was identified as the round-shaped area in the center of the brain. NAcc was isolated using punch dissection in 2 mm sections of brains (Alto™ brain matrix, Stoetling Co., Wood Dale, IL, USA) and identified under a stereomicroscope (Model SZX7, Olympus America Inc., Center Valley, PA, USA). The NAcc was identified as the tissue in the vicinity of the anterior branch of the anterior commissure, according to stereological coordinates [28]. Samples were frozen overnight at –20°C after adding perchloric acid 0.2 M. Samples were briefly sonicated and centrifuged, and 50 µL aliquots of the supernatant was injected on a high-performance liquid chromatography combined with electrochemical detection system. A mobile phase of 0.7 M aqueous potassium phosphate (monobasic) (pH 3.0) in 10% methanol, 1-heptanesulfonic acid (222 mg/L) and ethylenediaminetetraacetic acid disodium salt (Na-EDTA) (40 mg/L) was used.

Levels of 5-HT, 5-hydroxyindoleacetic acid, dopamine, 3,4-dihydroxyphenylacetic acid, and 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid) were determined using a Gilson instrument (Gilson Inc., Middleton, WI, USA), fitted with an analytical column (Supelco Supelcosil LC-18 3 M; 7.5 cm × 4.6 mm; flow rate: 1.0–1.5 mL/min; Supelco, Bellefonte, PA, USA). A standard curve was then obtained and data presented as concentration (nanogram per milligram of tissue protein).

Molecular Analysis

For real-time PCR analysis, total RNA was isolated from frozen areas using Trizol (Invitrogen, Heidelberg, Germany) and DNase treated (Fermentas, Vilnius, Lithuania), according to the manufacturer. Two micrograms of RNA were converted into cDNA using the iSCRIPT kit (Bio-Rad, Hercules, CA, USA). RT-PCR was performed using SyberGreen (Qiagen, Valencia, CA, USA) and the Bio-Rad q-PCR CFX96 apparatus. Hypoxanthine-guanine phosphoribosyltransferase (HPRT) was used as a housekeeping gene. We used relative quantification to determine the fold-change difference between control, CORT, and DEX animals, using the ΔΔCT method as described before [29]. Primer sequences were AR_F:GGGTGACT TCTCTGCCTCTG, AR_R:CCACAGATCAG GCAGGTCTT (AR); ESR1_F:CAGGTGCCCT ACTACCTGGA, ESR1_R:GGTAGCCAGAGG CATAGTCG (estrogen receptor 1); ESR2_F:AA CCGCCATGAGTATTCAGC, ESR2_R:GTAA CAGGGCTGGCACAACT (estrogen receptor 2); nNOS_F:GACAACGTTCCTGTGGTC CT, nNOS_R:GAAGAGCTGGTCCTTTGT GC (neuronal nitric oxide synthase [nNOS]); D1R_F:TCCTTCAAGAGGGAGACGAA, D1R_R:CCACACAAACACATCGAAGG (dopamine D1 receptor); D2R_F:CATTGTCTGGGTCCT GTCCT, D2R_R:GACCAGCAGAGTGACGA TGA (dopamine D2 receptor); HPRT1_F:GC AGACTTTGCTTTCCTTGG, HPRT1_R:TC CACTTTCGCTGATGACAC.

Statistical Analysis

For statistical analysis, the "n" of each experiment group was considered the number of litters from which individuals were derived. Two males per litter were averaged in order to represent each litter. Results are presented as average ± standard error. Data were analyzed by PASW Statistics 18.0 (SPSS Inc, Chicago, IL, USA), using analysis of variance. Whenever appropriate, post hoc comparisons were performed using Tukey's test; statistical significance was considered when $P < 0.05$.

Results

Acute Antenatal DEX Exposure Affects Adult Male Sexual Behavior

Treatment significantly affected sexual motivation and the volitive aspects of copulatory behavior ($F_{(2,9)} = 20.057$, $P < 0.001$), as revealed by an increased latency to mount in DEX-exposed animals compared to controls ($P < 0.001$) and CORT subjects ($P = 0.029$), respectively (Figure 2) [30]. CORT animals were also different from controls ($P = 0.027$).

Treatment also affected the number of mounts ($F_{(2,9)} = 5.233$, $P = 0.031$), another measure of sexual motivation [27], which was significantly decreased in DEX-exposed subjects when com-

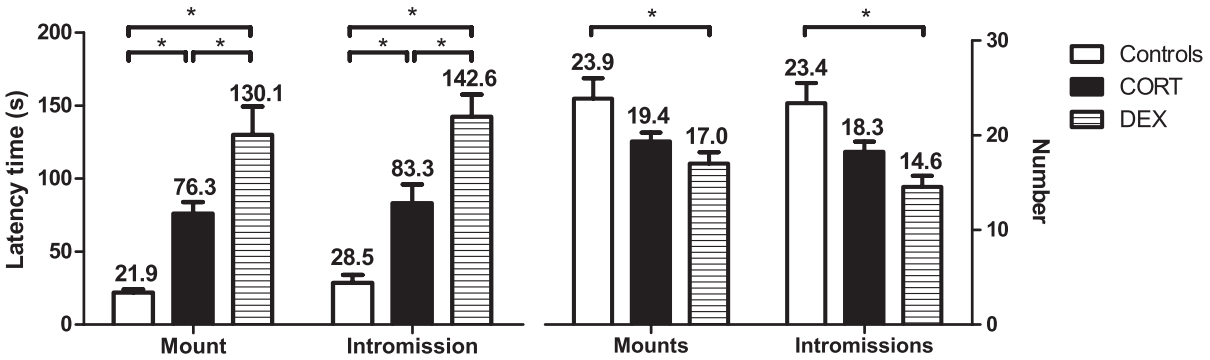

Figure 2 Mount and intromission latencies were increased in dexamethasone (DEX) and corticosterone (CORT) exposed animals when compared to controls (left); the number of mounts and intromissions was significantly reduced in DEX-exposed rats (right). *$P < 0.05$.

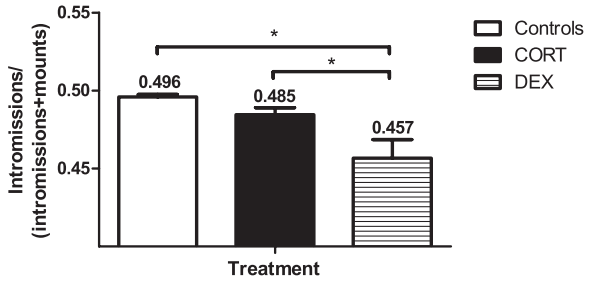

Figure 3 Intromission ratio, calculated as *intromissions/(intromissions + mounts)*, was diminished in dexamethasone (DEX)-exposed rats when compared to controls and corticosterone (CORT) animals. **P* < 0.05.

pared to controls ($P = 0.027$). Interestingly, CORT rats were not different from the other groups.

In contrast to mounts, intromissions are not exclusively dependent on sexual motivation [27]. Treatment affected this parameter ($F_{(2,9)} = 23.081$, $P < 0.001$) as revealed by an increased latency of DEX progeny in comparison to controls ($P < 0.001$) and CORT subjects ($P = 0.016$). Again, CORT animals also took more time to intromission than controls ($P = 0.024$).

Moreover, the number of intromissions was affected by treatment ($F_{(2,9)} = 8.083$, $P = 0.010$). This indicator of the easiness in the activation of ejaculatory reflexes [27] was found to be decreased in DEX-exposed ($P = 0.008$) but not on CORT-exposed subjects ($P = 0.103$).

Intromission ratio (Figure 3), an indicator of the efficiency of penile erection, was also affected by treatment ($F_{(2,9)} = 22.972$, $P < 0.001$) [27]. DEX-subjects displayed significantly lower ratios compared to controls ($P < 0.001$) and CORT-exposed animals ($P = 0.003$). Ejaculation number was similar between groups (data not shown).

Adult Testosterone Levels are Affected by Antenatal DEX

Serum testosterone levels were significantly reduced by antenatal DEX exposure ($F_{(2,9)} = 15.815$, $P = 0.001$; vs. CORT $P = 0.001$; vs. controls $P = 0.004$) but not by CORT (Figure 4). Testis wet weight was similar between groups (data not shown).

Antenatal Corticosteroids Influence Brain Dopamine Levels

Antenatal corticosteroids exposure influenced the levels of dopamine in NAcc ($F_{(2,9)} = 39.911$, $P < 0.001$; Table 1). Both progeny of CORT and DEX dams displayed decreased levels of dopamine in NAcc in comparison to controls ($P < 0.001$ and $P < 0.001$, respectively). Treatment also affected dopamine turnover ($F_{(2,9)} = 6.162$, $P = 0.021$), with an increase in DEX subjects when compared to CORT ($P = 0.026$) and controls ($P = 0.047$).

Hypothalamic levels of dopamine were also significantly affected by antenatal treatment ($F_{(2,9)} = 5.817$, $P = 0.024$), with a decrease only in DEX-treated subjects ($P = 0.022$) when compared to controls. Dopamine turnover in this area was also affected ($F_{(2,9)} = 10.286$, $P = 0.005$), with CORT and DEX animals displaying a lower ratio when compared to controls ($P = 0.010$ and 0.008, respectively).

Treatment did not affect serotonin levels ($F_{(2,9)} = 3.564$, $P = 0.072$) nor its turnover ($F_{(2,9)} = 1.076$, $P = 0.381$) in the NAcc. However, hypothalamic levels of serotonin were reduced ($F_{(2,9)} = 14.050$, $P = 0.002$), while its turnover was increased ($F_{(2,9)} = 7.552$, $P = 0.012$) in both DEX ($P = 0.003$; $P = 0.029$) and CORT ($P = 0.004$; $P = 0.016$) groups.

Molecular Correlates

In the NAcc, dopamine D1 (D1R) and D2 (D2R) receptors mRNA levels were significantly affected by prenatal exposure to corticosteroids ($F_{(2,9)} = 111{,}471$, $P < 0.001$ and $F_{(2,9)} = 76.383$, $P < 0.001$, respectively; Table 2), with an increase in DEX group when compared to controls ($P < 0.001$; $P < 0.001$, respectively) and CORT ($P < 0.001$; $P < 0.001$, respectively). A similar effect was also observed in the hypothalamus, but only for D1R ($F_{(2,9)} = 7.868$, $P = 0.011$; vs. controls $P = 0.009$); CORT animals were not different from any other group.

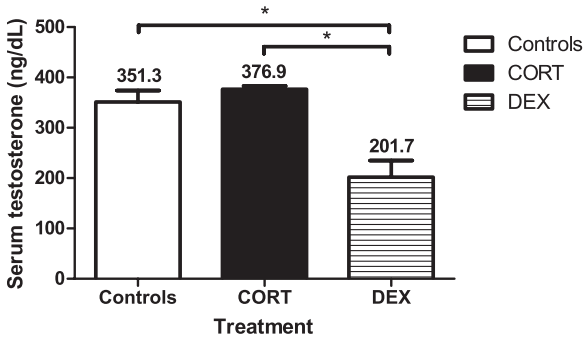

Figure 4 Antenatal dexamethasone (DEX) administration led to diminished serum testosterone levels (ng/dL) in adult male rats, when compared to corticosterone (CORT) and controls. **P* < 0.05.

Table 1 HPLC measurement of dopamine and serotonin (5-HT) levels and respective turnover in nucleus accumbens and hypothalamus

	Treatment	Dopamine	Dopamine turnover	5-HT	5-HT turnover
Nucleus accumbens	Controls	319.53 (22.24)*	0.785 (0.023)	62.17 (1.54)	0.170 (0.018)
	CORT	146.29 (0.76)	0.713 (0.030)	38.07 (4.19)	0.196 (0.915)
	DEX	162.17 (13.93)	1.333 (0.234)†	49.82 (10.12)	0.215 (0.028)
Hypothalamus	Controls	21.46 (2.82)	1.212 (0.095)*	30.31 (1.72)*	0.030 (0.014)*
	CORT	15.33 (1.10)	0.825 (0.010)	22.34 (0.80)	0.075 (0.003)
	DEX	12.82 (1.03)‡	0.808 (0.078)	22.18 (1.01)	0.070 (0.007)

Values represent mean (SE). *Different from CORT and DEX ($P < 0.05$); †different from controls and CORT ($P < 0.05$); ‡different from controls ($P < 0.05$).
Dopamine and serotonin (5-HT) tissue concentration (ng/mg total protein) and respective turnover. Dopamine turnover assessed by the ratio between dopamine metabolites and dopamine concentrations; Serotonin (5-HT) turnover assessed by the ratio between 5-hydroxyindoleacetic acid (5-HIAA) and 5-HT concentrations.
HPLC = high-performance liquid chromatography; CORT = corticosterone-exposed animals; DEX = dexamethasone-exposed animals.

Levels of AR and nNOS mRNA were not different between groups in the NAcc. However, in hypothalamus, there was a significant effect of exposure on AR ($F_{(2,9)} = 13.049$, $P = 0.002$) and nNOS ($F_{(2,9)} = 27.056$, $P < 0.001$) mRNA levels, with an increase in DEX progeny when compared to controls ($P = 0.020$; $P < 0.001$, respectively) and CORT subjects ($P = 0.002$; $P = 0.002$, respectively).

Levels of estrogen receptors 1 and 2 were not affected by treatment in neither area.

Discussion

In rats, brain sexual differentiation occurs mainly during late gestation (ED 14–21) and the first 2 weeks of postnatal life [1]. The vulnerability of this time-window to several insults, including stress and corticosteroid exposure, may thus lead to long-term consequences on adult male sexual behavior [2,3]. In fact, previous data suggest that prenatal stress disrupts the normal maternal hormonal milieu and suppresses the fetal testosterone peak on ED 18 and 19, required for later expression and maintenance of male sexual behavior [31,32]. Such data sustain that interferences in the maturation of the HPA axis, affect the hypothalamic–pituitary–gonadal axis, since both are regulated by common players both centrally and at the periphery [5]. Following the initial experimental evidence showing that late gestation whole-body restraint under bright lights results in delayed initiation of copulation [33], more recent data correlated prolonged prenatal stress/corticosteroids with impaired adult male sexual behavior and reduced serum testosterone levels [2,3]. However, no previous studies focused on the potential effects of a short-term exposure which better mimics the everyday clinical practice, nor in the comparison between different types of corticosteroids.

Appetitive behaviors are more variable than the relatively stereotypic consummatory behaviors and are thought to reflect an underlying, inferred motivational state, leading an organism into contact with a certain stimulus. Sexual motivation includes sensory and motor processes that may be sensitized by gonadal hormones, as well as more general activational processes that may affect other behaviors [34]. The analysis of several behavioral parameters permitted us to distinguish between appetitive and consummatory components of male rat sexual behavior. Male rat sexual behavior is characterized by a series of mounts, either with or without vaginal penetration (intromission), ultimately leading to ejaculation [35]. Latency until

Table 2 Gene expression analysis (real-time PCR) in nucleus accumbens and hypothalamus

	Treatment	D1R	D2R	AR	ESR1	ESR2	nNOS
Nucleus accumbens	Controls	1.023 (0.019)	1.010 (0.002)	0.96 (0.11)	0.97 (0.11)	0.96 (0.13)	0.96 (0.12)
	CORT	1.191 (0.067)	0.670 (0.015)	1.05 (0.11)	1.07 (0.05)	1.40 (0.21)	1.10 (0.04)
	DEX	2.131* (0.070)	1.779* (0.112)	1.33 (0.14)	1.09 (0.14)	1.00 (0.03)	1.23 (0.25)
Hypothalamus	Controls	1.010 (0.044)	0.944 (0.032)	0.949 (0.120)	0.888 (0.134)	1.088 (0.116)	0.805 (0.162)
	CORT	1.230 (0.130)	0.820 (0.035)	0.779 (0.046)	1.358 (0.085)	1.259 (0.124)	1.133 (0.050)
	DEX	1.570† (0.106)	0.938 (0.031)	1.308* (0.011)	1.421 (0.189)	1.485 (0.179)	1.886* (0.147)

Values represent mean (SE). *Different from controls and CORT ($P < 0.05$); †Different from controls ($P < 0.05$).
The mRNA expression levels are presented as the fold change increase in relation to the respective control.
D1R = dopamine D1 receptor; D2R = dopamine D2 receptor; AR = androgen receptor; ESR1 = estrogen receptor 1; ESR2 = estrogen receptor 2; nNOS = neuronal nitric oxide synthase; CORT = corticosterone-exposed animals; DEX = dexamethasone-exposed animals.

the first mount reflects some of the appetitive aspects and sexual motivation; on the other hand, intromission and ejaculation latencies, but also mount and intromission frequencies, reproduce consummatory components of copulatory behavior [30,36]. Interestingly, the impairment of male sexual behavior observed in this study was mainly characterized by alterations in sexual motivation (increase in mount and intromission latencies) [34]; moreover, the reduced number of mounts might also reflect decreased sexual motivation [27]. Importantly, these differences are more striking in animals exposed to DEX than to CORT.

Regarding the neurobiology of sexual behavior, three major integrative systems regulate sexual motivation and genital and motor responses [8]. Whereas the mesolimbic system is critical for appetitive behavior and reinforcement, the medial preoptic system contributes to genital reflexes, sexual motivation, and motor patterns of copulation. Finally, the nigrostriatal system enhances the motoric readiness to respond to stimuli. Dopamine is the common key player in all three systems, easing sexual motivation, copulatory proficiency, and genital reflexes [7]. The pathways for sexual excitation involve the activation of incertohypothalamic and mesolimbic dopamine transmission that targets the hypothalamic medial preoptic area (MPOA) and NAcc, respectively [37]. As a result, in the male rat, there is a slight increase in dopamine release in NAcc following presentation to a receptive female that is followed by a sharp increase in dopamine transmission during copulation, which gradually declines after the removal of the female [38].

In prenatally stressed male rats, the absence of copulatory behaviors is associated with a failure to increase extracellular levels of dopamine and its metabolites in the NAcc. Such data, obtained through simultaneous sexual behavior testing and concomitant microdialysis sampling, suggested that intense environmental stressors might impair NAcc dopamine release [39].

In our experiment, an interesting neurochemical–behavioral link was established. Serotonin levels in the NAcc and hypothalamus were not increased in CORT or DEX progeny when compared to controls, suggesting that the sexual impairment observed is unlikely to be related to the inhibitory effects of serotonin [8,10,40]. On the other hand, we found decreased dopamine in hypothalamus and NAcc of animals briefly exposed to prenatal corticosteroids. Interestingly, we had previously reported a reduced dopaminergic innervation of the NAcc following prenatal short-term exposure to DEX, revealed by a reduced density of tyrosine hydroxylase-positive fibers in these subjects [6]. In addition, the decrease in dopamine levels in NAcc herein reported is likely to be of relevance for the changes in sexual behavior if one takes into account descriptions correlating a delayed onset of copulation and ejaculation with a diminished release of this neurotransmitter in the mesolimbic tract [8]. Furthermore, the increase in dopamine D1 and D2 receptors mRNA in the NAcc following DEX exposure herein shown further supports the existence of a hypodopaminergic status in these animals, and may appear as a compensatory mechanism due to the low dopamine levels observed.

Although we observed substantial differences in the dopamine levels and receptors in the NAcc, one limitation of this work is the fact that we did not discriminate between its two functionally distinct regions: the core and the shell. In fact, these two regions of the NAcc display distinct c-fos expression during sexual behavior; whereas there is increased c-fos in the core, its expression remains unchanged in the shell [41]. On the contrary, administration of drugs of abuse results in increased dopamine levels in the shell of the NAcc [42–45]. This suggests that shell and core might be activated differently in response to natural reinforcers and drugs of abuse. Indeed, NAcc neurons exhibit similar neuronal activity when responding to two natural rewards—food and water, but different firing patterns when responding for a natural reward vs. cocaine [46]. Therefore, considering the different functional/activational roles of core and shell, it would be interesting to analyze dopamine metabolism and receptors in each subarea in order to dissect what is the most affected area.

The hypodopaminergic status of DEX-exposed animals in the NAcc might have other behavioral consequences besides altered sexual behavior, considering the importance of correct dopamine input for feeding, reward, and addiction, among others. Dopamine is released in the NAcc in response to not only drugs of abuse, but also to other consummatory behaviors such as sex and food; thus, the ventral tegmental area (VTA)-NAcc pathway is also known as the "reward pathway" [47]. Interestingly, some studies have reported cross-sensitization between repeated exposures to pharmacological agents and natural motivated behaviors such as sex [48–50]. For example, sexual experience can cross-sensitize neuronal responses

to amphetamine and this seems to be dependent on dopamine release in the NAcc [41].

The intricately regulated balance between hypo- and hyperdopaminergic states in the mesolimbic circuit, especially in the NAcc area, underlies an individual's cycles of drug-seeking behavior/abuse and response to natural rewards. While a hyperdopaminergic state seems to enhance the motivational or rewarding properties of drugs of abuse, hypodopaminergic states appear to enhance drug-seeking behavior in parallel with reductions in the perceived motivational impact of "natural" rewards such as food and sex [51–53]. This theory is in agreement with our behavioral and neurochemical results, given the fact that DEX animals have low dopamine levels in the NAcc and, concomitantly, impaired appetitive sexual behavior. Additionally, it suggests that these animals might also display differential susceptibility to addiction, a phenomenon also observed in other models of early life stress [54].

Dopamine in the hypothalamus, particularly in the MPOA, is essential for genital reflexes, motor patterns of copulation, and probably sexual motivation [55]; several studies described the facilitative role of increased levels in the MPOA on sexual behavior, suggesting that testosterone might mediate this effect [56]. In the present study, conclusions on the impact of prenatal exposure to natural vs. synthetic corticosteroids are limited by the fact that dissection of the whole hypothalamic area was performed instead of isolating the MPOA. Nonetheless, the decreased hypothalamic levels of dopamine herein reported in the DEX group, but not in the CORT group, is associated with a significant effect on D1 receptors mRNA. This fact is likely to be of significance to explain the differential neuroendocrine and behavioral effects of CORT from DEX.

Since testosterone can directly activate AR, or indirectly via stimulation of estrogen receptors due to its aromatization to estradiol [57], we also analyzed the levels of these receptors in DEX and CORT progeny. Interestingly, an increase in AR mRNA was observed in the hypothalamus of DEX progeny, possibly reflecting a reduction in circulating androgens, but no differences were found in estrogen receptors. Interestingly, previous studies showed that although normal basal levels of dopamine in the MPOA are adequate to allow some copulatory behavior, efficient mating requires an androgen-dependent female-stimulated increase [58]. Also, by upregulating nNOS in the MPOA, testosterone enhances nitric oxide production, which controls dopamine release [59]. In the present study, we found increased levels of nNOS mRNA in the hypothalamus of DEX subjects. It would be of added value to assess if theses changes persist in MPOA samples, which would be in accordance to previous descriptions in the MPOA of gonadectomized rats [60]. However, technical issues in the isolation of the MPOA and the fact that neighbor hypothalamic subareas might display different susceptibilities to circulating androgens could justify why other studies did not confirm the original findings [61].

Thus, in order to draw further conclusions on the impact of the in utero corticosteroids exposure on the adult male MPOA, it would be of interest to specifically analyze this hypothalamic area in future studies.

Conclusions

Early life exposure to short-term glucocorticoid ligands triggers lifelong programming effects in brain regions implicated in distinct aspects of male sexual behavior. The behavioral changes are paralleled with altered dopaminergic systems and neuroendocrine markers. These findings are of clinical relevance, as they provide support to therapeutic interventions for sexual dysfunction that modulate brain dopaminergic levels [62–65] and peripheral levels of testosterone [66–68]. Noticeably, equipotent CORT administration triggers a less detrimental impairment than DEX, highlighting the role of the different corticosteroid receptors on the systems regulating sexual behavior. Now, it is important to dissect and distinguish how exposure to different glucocorticoids modulates the neuroendocrine, neurochemical, and molecular environment and to identify the changes directly relevant for the observed impairment in sexual behavior.

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Corresponding Author: Mário Oliveira, MD, Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Campus de Gualtar, Braga 4710-057, Portugal. Tel: (351) 253-604-929; Fax: (351) 253-604-820; E-mail: mariooliveira@ecsaude.uminho.pt

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Statement of Authorship

Category 1

(a) Conception and Design
Mário Oliveira; Nuno Sousa

(b) Acquisition of Data
Mário Oliveira; Pedro Leão; Ana-João Rodrigues; José-Miguel Pêgo

(c) Analysis an Interpretation of Data
Mário Oliveira; Nuno Sousa; Ana-João Rodrigues

Category 2

(a) Drafting the manuscript
Mário Oliveira

(b) Revising it for intellectual content
Ana-João Rodrigues; João-José Cerqueira; Nuno Sousa

Category 3

(a) Final approval of the completed manuscript
Mário Oliveira; Nuno Sousa

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Chapter 3

Discussion

3. DISCUSSION

The present work aimed to assess the programming effects of the acute prenatal exposure to corticosteroids in the adult brain and its impact on anxiety and fear behaviors. Initially, a behavioral characterization was performed (Chapter 2.1); this first study revealed a hyperemotional phenotype in animals treated with prenatal dexamethasone (DEX). Thus, in a subsequent study we performed a structural, neurochemical and molecular analysis of brain regions associated with the control of these functions (BNST and amygdala) (Chapter 2.2). In addition, given previous data from our lab showing an impoverished dopaminergic innervation of the mesolimbic pathways (Leao et al., 2007; Appendix 1), an assessment of adult male addictive behavior (Appendix 2) and sexual behavior was performed; the latter was correlated with neurochemical and molecular data from hypothalamus and nucleus accumbens (Chapter 2.3).

3.1. Animal model of antenatal corticosteroid exposure

The glucocorticoid (GC) milieu, both *in utero* and in the early postnatal period, is crucial for neurodevelopment not only because of its activational effects, but also through its important brain programming effects. Increasing evidence suggests that early life exposure to GC triggers undesired metabolic, cardiovascular, neuroendocrine and behavioral phenotypes in adulthood (Mesquita et al., 2009; Appendix 3). In this work, we aimed to understand the effects of a clinical relevant dose of GCs administered during pregnancy in a short-term basis. Thus, in our animal model of prenatal short-term exposure to GCs, we decided to use 1mg/kg of DEX, a dosage that was previously shown to have physiological effects in rodents (De Souza and Adlard, 1973).

To compare the effects of synthetic versus natural corticosteroids we also used in some of our studies a short-term administration of corticosterone (CORT) to pregnant dams. There is a paucity of studies regarding the dosages of CORT needed to exert similar physiological effects as DEX; however, given that DEX is 25 times more potent than CORT on GC receptor binding, we have used a 25mg/kg dose of CORT in our experiments. Importantly, both drugs (at the dosages used) seem to have similar physiological effects in both humans and rodents. In fact, animal studies have confirmed the efficacy of synthetic and natural corticosteroids in these relative dosages in promoting fetal lung maturation (Jobe et al., 2003). Moreover, human data supports the efficacy of both drugs in promoting lung maturation (Dluholucky et al., 1976; Roberts and Dalziel, 2006).

It is relevant to note at this point that the neurodevelopment processes in rodents and humans are not totally coincident. In contrast to humans, in which the phase of maximal fetal brain growth begins at 28–36 weeks (80% of gestation), maximal brain growth in rats does not occur until the first postnatal week (Dobbing and Sands, 1970, 1979). Therefore, in order to implement a model of late gestational exposure to GCs, pharmacological administration was performed during the final days of gestation, in order to better assess the effects on the developing fetal brain. At this point, it is important to highlight that our model proved to be efficient as it induces activational effects (e.g. differences in body weight) but also triggers relevant brain programming effects (see below) that persist throughout adulthood.

3.2 Behavioral assessment of anxiety and BNST structural correlates

The studies presented (Chapters 2.1 and 2.2) demonstrate that antenatal exposure to DEX results in increased anxiety-like behavior in adulthood. These conclusions are supported by data collected from different behavioral tests: two tests based on conflict behavior, the elevated plus maze (EPM) and the open-field (OF) tests, but also in a behavior paradigm, the acoustic startle response (ASR), that is independent on the animals' motivation to explore new contexts.

Among the different tests used to measure anxiety in rodents, the EPM has been thoroughly validated by both physiological and pharmacological measures (Pellow et al., 1985; Lister, 1987; Cruz et al., 1994; File, 2001), being considered by many as the gold-standard test to measure anxiety in rodents. The test recreates natural stimuli known to induce anxiety in humans, which are considered to be ethologically valid. In the EPM, the animals face the conflict of exploring a novel and well lit open narrow platform raised above the floor or to stay on a darker narrow alley with high walls protecting from potential threats (Dawson and Tricklebank, 1995). When tested in the EPM, rats exposed to anxiolytic drugs spend an increased percentage of time and entries in the open arms, reflecting the value of the test in the assessment of anxiety rather than exploration. The EPM is considered to rely on neuronal pathways related with processment of diffuse stimuli and not with specific cues; these include the BNST, but not the amygdala (Davis et al., 1997; Pego et al., 2008).

In our work, decreased ratio of open arm time and number of open arm entries were observed in DEX exposed rats. Interestingly, a group of rodents exposed to the non-synthetic GC, CORT, displayed an intermediate phenotype, possibly reflecting a different susceptibility of the developing nervous system to the activation of different

corticosteroid receptors. Our findings are supported by previous studies on the exposure to DEX during the last third of gestation (Welberg et al., 2001), even though other authors failed to replicate these findings (Hauser et al., 2009). Besides different dosage (0,1mg/kg/day) and duration of drug exposure (last gestational week), the latter authors suggest that the anxiogenic effects of prenatal DEX could be more easily visible under stressful background conditions only, since, in their work, the test was performed under the dark phase of the light/dark cycle; according to them, light could emphasize a DEX induced hypersensitivity to stress.

Herein we showed that the hyperanxious phenotype triggered by prenatal short-term exposure to DEX is also evident in the acoustic startle, an expression of unconditioned emotional behavior. The startle is a fast protective mechanism of the organism in response to a sudden and intense stimulus, being considered an indicator of anxiety-like behavior (Koch, 1999). In this test, the reflex response of the organism to loud noises, characterized by a sequential contraction of the skeletal musculature, is recorded and reflects the activation of a trisynaptic circuit: 1) auditory inputs from several brainstem nuclei (dorsal cochlear nucleus, cochlear root nucleus, ventral cochlear nucleus, lateral superior olive and ventrolateral tegmental nucleus) are conveyed to a main sensorimotor interface, 2) the caudal pontine reticular nucleus, which then projects to 3) spinal motor neurons to trigger the motor response (Lee et al., 1996; Koch, 1999). Thus, the assessment of anxiety through the acoustic startle reflex, by characterizing the impairment of a specific neuronal circuit, is thought to be more precise and less influenced by environmental and experimental conditions known to interfere with the EPM performance (Izidio et al., 2005; Lewejohann et al., 2006; Wahlsten et al., 2006). In our work (Chapter 2.2), DEX exposed subjects displayed increased startle amplitudes at different intensities and a more rapid increase in startle amplitudes as a function of stimulus

intensity, when compared to controls. Few studies had assessed the impact of prenatal GC exposure in the ASR, but recent data on the effect of prenatal DEX on female offspring (0.2 mg/kg, gestational days 14-21) also showed an increase in basal startle response, but only when an additional stressor was applied (e.g. by performing blood sampling three months before) (Hougaard et al., 2005; Kjaer et al., 2011).

Of notice, it was demonstrated that the response of stressors upon the ASR is modulated by a direct projection from the BNST to the caudal pontine reticular nucleus, but also by an indirect one via the CeA (Davis et al., 1997). Thus, the BNST and the CeA are the common regions of the brain that might influence the phenotype observed in the EPM and in the ASR in animals treated with prenatal GCs. Taking that into account, we decided to perform a better characterization of these brain regions in order to understand the underlying mechanisms of the DEX-induced programming of anxiety behavior. Moreover, it is well established the role of anxiogenic stimulus in the autonomic activation, including a stimulation of the HPA axis and subsequent elevation of corticosteroid levels (File et al., 1988). Such adaptative response allows coping when novel and potential threatening situations are presented; however, a sustained activation of the HPA axis may disrupt these stress-responsive systems and result in a permanent activation of these systems. The BNST is one, probably the most important, of the relay stations that conveys inputs from stress-sensitive areas of the cortex and limbic system both to the HPA axis but also to brain stem nuclei implicated in several emotional behaviors (Herman and Cullinan, 1997); its role on HPA axis modulation is largely exerted through inhibition of the PVN.

Impact of prenatal DEX on BNST morphology

Taking into consideration the role of the BNST in anxiety, and namely its modulatory effects in the ASR through projections to the caudal pontine

reticular nucleus (Davis et al., 1997), and the control it exerts over the HPA axis, a neuromorphological assessment of the BNST was mandatory. Interestingly, antenatal exposure to DEX affected both the volume and dendritic arborization of the anteromedial division of the BNST (BNSTam). This topographic specificity must be highlighted particularly in stress-induced anxiety. In fact, there is substantial evidence for the presence of numerous cell bodies that synthesize corticotropin releasing factor (CRF) (Ju et al., 1989; Phelix and Paull, 1990; Sahuque et al., 2006), a mediator of anxiety and stress-like responses (Bale and Vale, 2004) in this division of the BNST. Moreover, the BNSTam projects intensely to brainstem and hypothalamic regions (Cullinan et al., 1993; Moga and Saper, 1994; Spencer et al., 2005; Dong and Swanson, 2006) modulating neuroendocrine, autonomic, and behavioral responses associated with preservation of homeostasis. Of particular relevance to this work are the BNSTam projections to the paraventricular nucleus of the hypothalamus (Dong and Swanson, 2006), which participates in the modulation of peripheral corticosteroid levels. In fact, while electrical stimulation of the BNSTam results in increased plasma corticosterone levels (Dunn, 1987), its lesion produces a decrease of the plasmatic levels of these hormones (Choi et al., 2007).

Herein we described an increase in BNST total volume, an effect largely due to increased volumes in anteromedial division of the BNST, without significant impact on total cell numbers. Additionally, a remodeling of dendritic arborizations was identified, with prenatal DEX exposure resulting in an increase of total dendritic length of neurons in the BNSTam when compared to controls; however, no effects were identified in spine densities. Our findings of a general hypertrophy of dendrites in the neurons of this brain area are in accordance with data from studies on the effects of stress in adulthood (Vyas et al., 2003; Pego et al., 2008). Interestingly, the plastic changes observed in the BNSTam of

DEX-exposed animals are paralleled with alterations in the expression levels of NCAM, a molecule important for neuronal plasticity (Nacher et al., 2002; Bisaz and Sandi, 2010). In addition, the increased levels of synapsin, a protein involved in the regulation of neurotransmitter release (Rosahl et al., 1995), are also suggestive of an increased neurotransmission, and possibly overactivation, of this brain region following antenatal DEX exposure. Such molecular changes confirm a stress-induced enhanced plasticity in these brain regions, as suggested by the increased dendritic arborization of these animals.

A brief note to highlight that besides the increased anxiety-behavior, animals exposed to DEX in pregnancy also displayed increased depressive-like behavior in adulthood (Chapter 2.1). It is known that increased anxiety may prompt individuals to develop depression (Strohle and Holsboer, 2003). The overlap between the emotional alterations observed in anxiety and depressive disorders (Hettema, 2008) is supported by animal studies (Bessa et al., 2009), and suggests shared psychopathological pathways for both behaviors. Interestingly, in this work (Chapter 2.1), we observed that despite prenatal exposure to either CORT or DEX did not affect the baseline performance in the forced swimming test (FST), these animals were more susceptible to the development of depressive-like symptoms following additional exposure to a chronic mild stress protocol during adulthood. These observations of increased immobility in the FST were subsequently replicated in other labs (Kjaer et al., 2010), but in some only in female progeny that was reared by DEX-exposed dams (Hauser et al., 2009). A final note to highlight that a very recent study showing that daily restraint stress during late gestation resulted in offspring depressive-like behavior later in life, demonstrated that this phenotype was associated with reduced dendritic complexity and spine density of neonatal-generated granule cells (Tamura et al., 2011). These data support the role of hippocampal

neuronal network disruption in prenatally induced depressive disorders, whose characterization could also be of interest in our research model.

At this point, it becomes clear that the exposure of pregnant dams to DEX induces a programming structural effect in the BNST of their progeny, which renders them more anxious and prone for depressive-like behavior in adulthood. It is also important to note that we also found a hyperresponsive HPA axis in these animals (Chapter 2.1) that led to increased levels of circulating corticosteroids in adulthood. This fact might be relevant to explain the hyperemotional phenotype, as the increase of circulating corticosteroids might further promote the activation of the BNST as it is known that these hormones are implicated in the neuroanatomical changes observed in this brain region (Pego et al., 2010).

3.3 Behavioral assessment of fear and correlates in the structure of the amygdala

Following the data supporting a hyperanxious phenotype in the adult offspring of short-term DEX exposed dams, we also decided to perform an assessment of fear conditioning in these animals (Chapter 2.2). Surprisingly, our work suggests that DEX exposure impairs fear conditioning in adulthood.

As a result of triggering a wide range of defensive behaviors, conditioned fear becomes a key survival-related function when a potential threat is presented to the individual. The most popular measures of conditioned fear include freezing and fear-potentiated startle (Fendt and Fanselow, 1999). Considering the neuronal circuits involved in the fear-potentiated startle, visual or auditory, but also shock, pathways project via the thalamus and insular or perirhinal cortex to the BLa. The latter then

projects to the CeA and MeA, which send outputs to the pathway of the acoustic startle in the brainstem. Specifically, the CeA either projects directly or via mesencephalic reticular formation and deep layers of superior colliculus, while the MeA sends indirect projections via ventromedial hypothalamus and ventral periaqueductal gray (Davis, 2006). Thus, the different projections to the caudal pontine reticular nucleus are responsible for the increase in startle amplitude induced by fear conditioning to a previous neutral stimulus. Previous data supports that while baseline startle amplitudes are not affected by interference with these connections, the response to fear conditioning is.

Our finding of impaired fear conditioning in adulthood following antenatal DEX exposure (Chapter 2.2) favors the hypothesis of defective amygdalar function and memory consolidation for emotionally arousing experiences. Supporting our findings, recent studies showed that prenatally stressed animals displayed a reduced ability to learn the predictive nature of a stimulus (Markham et al., 2010). Impairment of fear memory consolidation was also observed in maternally stressed male mouse offspring (from gestational day 8 to 20) (Lee et al., 2011). These findings, along with data showing an impairment on context-induced fear conditioning in adult male rats following early life stress (neonatal isolation between postnatal days 2-9) (Kosten et al., 2006), support that adverse early life events can, indeed, modulate fear conditioning. Conversely, other authors suggested an enhanced fear-like behavioral profile in adult male offspring from pregnant females submitted to immobilization from day 14 of pregnancy until birth (8–9 days), as they presented an impairment in fear extinction; however, such effect was only present if additional prolonged stress protocol was applied in adulthood (Green et al., 2011).

Impact on the morphology of the amygdala

Several studies have contributed to the elucidation of the role of the amygdala and its divisions, particularly the CeA and BLa, in fear. A disruption of fear-potentiated startle has been observed following different types of CeA lesion (Hitchcock and Davis, 1987; Campeau and Davis, 1995; Walker and Davis, 1997). Electrolytic lesions of the CeA completely blocked the expression of fear-potentiated startle in rats (Kim and Davis, 1993). Moreover, the differential role of the BNST and the amygdala was supported, as only light enhanced startle, but not fear-potentiated startle, was disrupted by inactivation of BNST (Walker and Davis, 1997; Gewirtz et al., 1998). Interestingly, inactivation of the BLa impaired both behaviors. Nonetheless, recent data suggests that the lateral division of the BNST may tonically inhibit the fear-potentiated startle, probably via projections to the CeA (Meloni et al., 2006).

Considering the established role of the amygdala in fear conditioning, and the observed impairment in fear behavior, a neuromorphological assessment of the amygdala appeared essential. According to our data (Chapter 2.2), brief prenatal DEX-exposure results in decreased volumes in the CeA and BLa (but not in the lateral division of the amygdala), without affecting the total cell numbers. Indeed, the volumetric reduction observed in these amygdalar divisions largely results from dendritic atrophy. It is interesting to note that the described dendritic atrophy in the BLa (but not in the CeA) is in accordance with previous observations on the effects of chronic unpredictable stress during adulthood (Vyas et al., 2002); however, others have failed to show any effect on the volumes of cerebral cortex, corpus callosum, hippocampus, dentate gyrus or amygdala following a single course of betamethasone at postnatal day one (Yossuck et al., 2006). Several technical issues may explain this divergence, namely the time of the exposure and the nature of insults, as different windows of vulnerability

to the programming effects of different stimuli seem to occur. In fact, the effects of stress in the neuronal morphology of specific brain regions are remarkably distinct throughout life. While chronic juvenile stress seems to induce a general hypertrophy on amygdalar dendritic arborization (Eiland et al., 2011), adult stress seems to have no impact (Pego et al., 2008). However, it is important to mention that others have reported an increase in dendritic growth in the BLa and no changes in the CeA (Vyas et al., 2002; Vyas et al., 2003) in response to stress. Obviously, these apparent divergent results illustrate the complexity of the structural phenomena taking place in the amygdala in response to stress/glucocorticoids and further studies are needed to better understand these differences.

The relevance of dopamine for emotional behavior

The next step in our work was to establish the neurochemical correlates for our behavioral and structural findings. Amongst several neurotransmitters, we decided to evaluate dopamine (DA) in light of previous findings of our laboratory in this (Leao et al., 2007; Appendix 1) and similar (Mesquita et al., 2009; Appendix 3) animal models, but also on its relevance for the function of the amygdala. Indeed, DA is believed to play a facilitative role in the amygdalar function, specifically via VTA efferents (Asan, 1997). *In vitro* studies support that dopamine modulates the excitability of amygdalar, namely BLa, neurons (Kroner et al., 2005) and it was demonstrated the blockade of fear-potentiated startle by lesion of the VTA (Borowski and Kokkinidis, 1996). The facilitator effect of DA on affective behaviors may be explained, at least partially, by its actions at the cellular level on BLa neurons. DA receptor activation could simultaneously facilitate the BLa output in response to strong inputs that cause spike firing, while suppressing weaker inputs via activation of GABAergic interneurons. In our work, the observation of a hypodopaminergic status in the amygdala following prenatal DEX-

exposure (Chapter 2.2) seems of particular relevance if we consider the impairments in fear memory observed in these animals. In fact, this is in line with previous observations in models of DA-deficient mice, in which restoration of DA levels in the BLa was required for the formation of fear-related memory (Fadok et al., 2010). According to our data, the decreased amygdalar levels of dopamine are associated with increased expression of D2 receptor mRNA in DEX-exposed subjects. This is consistent with recent data showing that conditioned fear is modulated by D2 receptor pathway connecting the VTA and BLa, where freezing behavior and increased dopamine levels in the BLa in response to a conditioned stimulus were both inhibited by intra-VTA administration of a D2-agonist (De Oliveira et al., 2011); moreover, according to the same study, intra-BLa injection of a D2-antagonist resulted in inhibition of FPS.

3.4 Implications of antenatal DEX-exposure for sexual behavior

The final behavior phenotype that proved to be altered in DEX-exposed animals was male sexual behavior. The most important period in brain sexual differentiation of rodents comprises the last gestational week and the first two weeks of postnatal life (Segarra et al., 1991), making it particularly vulnerable to insults, including stress and high corticosteroid exposure. Actually, it has been reported that prenatal stress disrupts the normal maternal hormonal milieu, and suppresses the fetal testosterone peak on gestational days 18 and 19 required for the expression of male sexual behavior in adulthood (Ward and Weisz, 1984; Lalau et al., 1990). An impact in the function of the hypothalamic-pituitary-gonadal (HPG) axis is suggested to occur as a result of disturbing the normal maturation of the HPA axis, as both axes share central and peripheral mechanisms of regulation (Page et al., 2001). In addition, previous studies reported that both prolonged prenatal stress

or corticosteroids-exposure result in reduced peripheral testosterone levels and impairments in adult male sexual behavior (Ward, 1972; Gerardin et al., 2005; Piffer et al., 2009), further supporting the need to clarify the potential effects of a brief prenatal corticosteroid exposure that better mimics the daily clinical practice.

When assessing male sexual behavior, two distinct patterns of behavior, initially described by Wallace Craig, are classically considered: 1) appetitive and 2) consummatory (Craig, 1917; Ball and Balthazart, 2008). While appetitive behaviors are variable and reflect the motivational status driving the individual to respond to a stimulus, consummatory behaviors are more stereotyped and species-specific (Pfaus, 1996; Hull and Rodriguez-Manzo, 2009). The analysis of several behavioral parameters differentiates the appetitive and consummatory components of male rat sexual behavior. Briefly, male rat sexual behavior is characterized by a succession of mounts, either with or without vaginal penetration (intromission), that eventually lead to ejaculation (Hull and Dominguez, 2007). Appetitive aspects and sexual motivation are inferred from latency to first mount (Hull et al., 2006), while consummatory components of copulatory behavior are reflected by intromission and ejaculation latencies but also mount and intromission frequencies (Pfaus et al., 1990a; Agmo, 1999).

According to our data (Chapter 2.3), brief prenatal DEX-exposure impaired mainly sexual motivation, as reflected by increased mount and intromission latencies, but also reduced number of mounts; notably, these differences were more striking in animals exposed to DEX than to CORT. Previous studies, using less detailed sexual behavioral assessment, reported impaired adult male sexual behavior following prenatal restraint stress (GD 18-22) (Gerardin et al., 2005) or aromatase inhibitor exposure (GD 21-22) (Gerardin et al., 2008). A transient impairment in male sexual behavior was also reported after

prolonged prenatal exposure to DEX or restraint stress, but not CORT (GD 14-21) (Holson et al., 1995); importantly, in the latter study the authors only measured "lordosis quotient" and ejaculations. While opposite results were reported by others following neonatal DEX-exposure (0,3mg/kg/day, from postnatal day 1-3), with animals showing increased sexual motivation and intromission behavior in the bi-level chamber (Kamphuis et al., 2004), the behavioral parameters assessed in our work are somewhat reproduced in a detailed report on the impact prolonged prenatal stress (GD 14-21) (Meek et al., 2006). Thus, in conclusion it seems that most reports in literature point to an impairment in male sexual performance after exposure to DEX in late pregnancy. This conclusion led us to search for the underlying mechanisms of this behavioral dysfunction, particularly the decrease in sexual motivation.

The hypodopaminergic status and its implications in the central control of sexual behavior

Taking into account the central role of the nucleus accumbens (NAcc) and DA in sexual behavior, particularly in sexual motivation, but also data from our group describing an impoverished dopaminergic innervation of the NAcc following brief prenatal DEX-exposure (Leao et al., 2007; Appendix 1), we decided to assess the impact of such exposure on the dopaminergic circuits in adulthood.

Dopamine (DA) is the common key player in all the three major integrative systems that regulate sexual motivation and genital and motor responses (Hull et al., 2004). This neurotransmitter favors sexual motivation, copulatory proficiency, and genital reflexes (Giuliano and Allard, 2001). Among these systems: 1) the mesolimbic system is critical for appetitive behavior and reinforcement; 2) the medial preoptic

system contributes to genital reflexes, sexual motivation and motor patterns of copulation; and 3) the nigrostriatal system enhances the motoric readiness to respond to stimuli. The neuronal circuits underlying sexual excitation involve the activation of incertohypothalamic and mesolimbic dopamine transmission that targets the hypothalamic medial preoptic area (mPOA) and NAcc, respectively (Pfaus, 2009). Therefore, during male rat sexual behavior increased DA release is observed in the NAcc. After a small augment following presentation of a receptive female, a sharp increase in dopamine transmission is observed during copulation, that steadily declines on female removal (Pfaus et al., 1990b).

In our work (Chapter 2.3), we established an interesting neurochemical link with the findings in impaired sexual behavior of the male GC-exposed progeny, as we reported decreased dopamine in hypothalamus and NAcc of animals briefly exposed to prenatal corticosteroids. This is in accordance with a prior simultaneous behavioral and microdialysis sampling study linking the absence of copulatory behaviors in prenatally stressed male rats who failed to increase extracellular levels of dopamine and its metabolites in the NAcc (Wang et al., 1995), thus supporting the potential impact of intense environmental stressors in NAcc dopamine release. The findings of decreased dopamine levels in the NAcc are also pertinent if we consider descriptions correlating a delayed onset of copulation and ejaculation with a diminished release of this neurotransmitter in the mesolimbic tract (Hull et al., 2004).

Apparently, these changes are related to a general impoverished of the dopaminergic innervation of the NAcc as suggested by our observations of decreased density of tyrosine hydroxylase-positive fibers in this area following prenatal short-term exposure to DEX (Leao et al., 2007; Appendix 1). The existence of a hypodopaminergic status in these animals is further supported by the report of increased dopamine D2

receptors mRNA in the NAcc, possibly as a compensatory mechanism for the low dopamine levels (Appendix 2). Considering the different response of the two regions comprising the NAcc, core and shell, to natural reinforcers, it would have been of interest to assess the differential impact of DEX-exposure on them. In fact, whereas increased c-fos expression is observed in the core of the NAcc during sexual behavior, it remains unchanged in the shell (Bradley and Meisel, 2001).

The mesolimbic reward circuit, in which the VTA-NAcc pathway is the central component, is an essential element when understanding the impact of the prenatal DEX-induced hypodopaminergic status on behavior. In fact, dopamine is released in the NAcc in response to consummatory behaviors such as sex and food, but also drugs of abuse (Piazza and Le Moal, 1996). Therefore, besides impaired sexual behavior, other behavioral consequences should be expected, since adequate dopamine input to the NAcc is essential for feeding, reward and addiction. In fact, the individual's cycles of drug-seeking behavior/abuse and response to natural rewards are tightly regulated by the balance between hypo- and hyperdopaminergic states in the mesolimbic circuit, and particularly in the NAcc. Therefore, while motivational or rewarding properties of drugs of abuse appear to be potentiated by hyperdopaminergic states, hypodopaminergic states seem to increase drug-seeking behavior along with a decline in the motivational impact of 'natural' rewards such as food and sex (Diana et al., 1993; Diana et al., 1998; Melis et al., 2005). The reduced dopamine levels in the NAcc and concomitant impaired appetitive sexual behavior observed in the DEX-exposed progeny further support this hypothesis. In addition, as reported in other models of early life stress (Kippin et al., 2008), it would also be also reasonable to assume a differential susceptibility to addiction. Actually, our group recently reported an increased preference for opiates and ethanol, along with simultaneous hyperresponsiveness to the psychostimulatory actions of morphine

following brief prenatal exposure to DEX (Appendix 2). These behavioral findings are in accordance with previous studies showing cross-sensitization between repeated exposures to pharmacological agents and natural motivated behaviors such as sex (Mitchell and Stewart, 1990b, a; Fiorino and Phillips, 1999).

Besides its role in the mesolimbic circuit, DA in the hypothalamus, particularly in the mPOA, is vital for genital reflexes, motor patterns of copulation, and (probably) sexual motivation (Hull and Dominguez, 2006). Thus, and despite our data relies on the whole hypothalamic area, and not the mPOA specifically, the decreased hypothalamic levels of dopamine exhibited following DEX might be of relevance. Of notice is the fact that antenatal CORT-exposure did not produce a similar reduction in hypothalamic DA levels nor had impact on D1 receptors mRNA levels, which might be relevant to explain the differential neuroendocrine and behavioral effects of CORT from DEX.

Given that the facilitative role of increased levels of DA in the mPOA on sexual behavior is considered to be mediated by testosterone (Dominguez and Hull, 2005), which can activate androgen receptors, either directly or via stimulation of estrogen receptors following aromatization to estradiol (Stewart and Rajabi, 1994), we also assessed the levels of androgens and its receptors in the hypothalamus. We found an augment in androgen receptor mRNA in the hypothalamus of DEX progeny, which possibly reflects the decreased circulating androgens; interestingly, estrogen receptors were unaffected. These findings are of relevance as an androgen-dependent female-stimulated DA raise is required for efficient mating (Putnam et al., 2005). Furthermore, testosterone is considered essential to regulate nNOS transcription in the mPOA, with the consequent influence in nitric oxide production, and modulation of DA release (Sanderson et al., 2008).

While it is clear the involvement of changes in the DAergic circuits and of hypothalamic androgens as underlying causes of the altered sexual behavior displayed by the adult male DEX-exposed progeny, there are several aspects that need further clarification to better understand this dysfunctional behavior, namely a greater topographic and molecular specificity of the reported changes in the NAcc and, mainly, in the hypothalamus. These are some of the aspects currently under analysis in our lab.

3.5 The selective effect of antenatal DEX exposure

At this point, one might wonder if all behaviors would be affected by antenatal DEX-exposure, or whether there is some specificity in the behavioral changes. Certainly, the latter seems to be the answer. Indeed, in the present work, we failed to show any deficit in spatial memory (Chapter 2.1). Such fact supports the view that brain structures may be subject to differential programming by corticosteroids during specific time windows; in this specific case it seems that neuronal circuits regulating emotionality (primarily, the BNST and amygdala) are vulnerable, whereas spatial memory systems (primarily, the hippocampus) are not. However, other authors described impaired spatial learning following prolonged prenatal stress (Markham et al., 2010) or DEX-exposure (Brabham et al., 2000), during the last gestational week (and not E18 and E19, as we did); moreover, in the latter study, changes were only observed in subjects reared by exposed mothers, possibly due to altered maternal behavior. Nonetheless, data from our lab does not confirm the presence of major changes in pup-directed or self-directed behaviors following short-term DEX-administration (Appendix 2). It is also relevant to note, that studies from our lab also demonstrate cognitive deficits if rats are exposed to stress/GCs in the first week of postnatal life (Mesquita et al., 2009;

Appendix 3). Thus, it seems most likely that the selective impact of prenatal GCs exposure on emotional and sexual behavior, without apparently affecting cognition, is due to the specific time period of the exposure to DEX, an aspect that can be ascribed to the different temporal maturation of brain circuits namely in what regards the expression of GR and MR (Rosenfeld et al., 1993; Owen and Matthews, 2003).

Also noticeable is the absence of changes in the prepulse inhibition test following DEX exposure (Chapter 2.2). In this test, which assesses the sensorimotor gating mechanisms, the amplitude of startle response is reduced when a non-startling acoustic stimulus is previously presented; due to its nature, this test is considered of relevance to schizophrenia. Acoustic prepulses are processed via ascending auditory pathway, including the inferior colliculus and then activating the superior colliculus, which also receives other acoustic, tactile and visual inputs. Projections from the latter to the pedunculopontine tegmental nucleus mediate the inhibition of startle response through a cholinergic projection to the caudal pontine reticular nucleus (Fendt et al., 2001). The absence of an effect on acoustic startle response to inhibitory prepulse described in our study is in accordance with previous data (Hauser et al., 2006), and suggests that GR antenatal programming is not of clear relevance to the neurodevelopmental hypothesis of schizophrenia.

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Chapter 4

Conclusions and Future Perspectives

4. CONCLUSIONS AND FUTURE PERSPECTIVES

In summary, we report the deleterious effects of prenatal exposure to DEX in emotional behavior, namely fear and anxiety. Moreover, neuromorphological, neurochemical and molecular programming changes in the brain areas related with these behaviors, namely in the amygdala and in the BNST, seem to underlie the hyperanxious and fear pathology in adulthood. These findings are also associated with the reprogramming of the HPA axis, leading to a hyperresponsive axis. Notably, the impact on emotional behavior appears to extend beyond anxiety and fear, being associated with increased susceptibility to develop depressive-like behavior following exposure to chronic stress.

In parallel, we demonstrate an impairment in adult male sexual behavior, mainly in its motivation-related aspects. This behavioral dysfunction was also associated to a hypodopaminergic status in both the NAcc and the hypothalamus. In the latter region, we also found changes in androgen signaling.

Interestingly, despite not being the main purpose of this thesis, our data points out the possibility of a less deleterious impact of the exposure to natural GCs, namely corticosterone. These findings, with potential clinical implications, further support the concerns raised on the impact of the excessive or repeated prenatal use of synthetic corticosteroids and call for the need of a re-appraisal of its therapeutic regimens and certainly for its parsimonious use.

As in every scientific work, at the end there seems to be more questions to be addressed than answers given... this is the driving force of science. Future work should aim the establishment of functional correlates of the described behavioral and neuromorphological phenotypes. Also, the impact of the HPA axis impairment on specific neuronal networks involved in emotional behavior should be further clarified by additional

molecular and neurochemical studies on its main neurotransmitter systems.

Several strategies could be considered to achieve these goals:

1) The behavioral, structural and molecular findings that emphasize the changes in BNST functionality could benefit from electrophysiological studies in this region.

2) The regulatory influence of the BNST on the HPA axis via PVN projections should be further clarified, namely through molecular studies on CRF receptors and tracer studies on CRF neurons. In parallel, the study of the GABAergic system, the main neurotransmitter system of the BNST, could further elucidate the regulatory influence this region over the HPA axis.

3) The described decrease in amygdalar DA levels together with the increased D2 receptor gene expression should be further explored. Tracer studies on the dopaminergic input to the BLa from the VTA could be of interest.

4) Given the described impairment in motivation-related aspects of sexual behavior, detailed neurochemical studies on NAcc core and shell and mPOA could better clarify the impact of prenatal exposure to corticosteroids in the brain dopaminergic systems.

5) The addictive phenotype reversion evidenced by L-dopa administration (Appendix 2) highlights the potential role of the dopaminergic system at least in part of the observed changes, namely in male sexual behavior. Would the observed impairments in sexual motivation also be reverted by L-dopa administration?

And which side effects could be expected from its long-term administration?

Hopefully, in a near future answers to these questions will be available. I hope to be able to contribute to the elucidation of some of them.

Chapter 5

Appendix

Appendix 1

Leão P, Sousa JC, **Oliveira M**, Silva R, Almeida OF, Sousa N

Programming effects of antenatal dexamethasone in the developing mesolimbic pathways

Synapse 61:40-49

(2007)

SYNAPSE 61:40–49 (2007)

Programming Effects of Antenatal Dexamethasone in the Developing Mesolimbic Pathways

P. LEÃO,[1] J.C. SOUSA,[1] M. OLIVEIRA,[1] R. SILVA,[1] O.F.X. ALMEIDA,[2] AND N. SOUSA[1]*
[1]*Life and Health Sciences Research Institute (ICVS), University of Minho, Campus de Gualtar, Braga, Portugal*
[2]*Neuroadaptations Group, Max Planck Institute of Psychiatry, Munich, Germany*

KEY WORDS nucleus accumbens; ventral tegmental area; neurogenesis; dopamine (DA); stereology

ABSTRACT Elevated glucocorticoids, during pregnancy, alter emotionality and increase propensity to drug abuse later in life, albeit through substrates and mechanisms are largely unknown. In this study, we examined whether antenatal glucocorticoid exposure induces enduring structural changes in the nucleus accumbens (NAcc), an important relay point in the reward limbic circuitry. To this end, rat dams were exposed to the synthetic glucocorticoid dexamethasone (DEX) on days 18 and 19 of gestation, and stereological tools were used to assess the total volume of, and neuronal numbers in, the NAcc, as well as the density of mesencephalic dopaminergic inputs from the ventral tegmental area (VTA) to the NAcc in their adult offspring. Further, we used measures of bromodeoxyuridine incorporation into NAcc cells to examine whether DEX-induced effects on cell proliferation represent another mechanism through which glucocorticoids alter the structure of mesolimbic pathways and might influence addictive behavior. Our studies show that exposure to DEX during late gestation results in significantly reduced volumes and cell numbers in the NAcc. The latter measure correlated strongly with a reduced rate of cell proliferation in DEX-exposed animals. Moreover, the treatment resulted in a decreased number of cells expressing tyrosine hydroxylase in the VTA and an impoverished dopaminergic innervation of the NAcc. These observations, which identify glucocorticoid-sensitive structures and neurochemical targets within the developing ''reward pathway,'' pave way for future studies designed to understand how early life events can predispose individuals for developing drug dependence in adolescent and adult life. **Synapse 61:40–49, 2007.**

INTRODUCTION

The fetus is highly sensitive to perturbations of its chemical environment during critical developmental windows. However, the fetal brain is not only sensitive to teratogenic agents; for example, exposure to high levels of adrenal corticosteroids (CSs) can set long-term programs in behavior by altering brain chemistry and morphology. As we recently demonstrated in rats, pharmacologically induced hypercorticalism during late pregnancy leads to increased emotionality, a feature which persists throughout life (Oliveira et al., 2006). Moreover, exposure to excess levels of CSs during late fetal stages are thought to lead to increased susceptibility to a variety of neurological and psychiatric disorders during childhood and adult life (Boska and El-Khodor, 2003; Lewis and Levitt, 2002; Weinstock et al., 1988), including increased susceptibility to drug abuse (Meaney et al., 2002; Thadani et al., 2004). The latter is thought to be associated to disturbances in either emotional or mood states, or both (Regier et al., 1990).

The nucleus accumbens (NAcc) is a heterogeneous structure belonging to the ventral division of the striatum. Histological and neurochemical studies reveal two anatomically distinct regions: a core and a shell (Zahm and Brog, 1992). Both core and shell

*Correspondence to: Nuno Sousa, Escola de Ciências da Saúde, CP II Piso 3, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal. E-mail: njcsousa@ecsaude.uminho.pt

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receive inputs from the amygdala, globus pallidus, and ventral pallidum. However, they differ in the density of their cortical afferents; the core receives projections predominantly from the prelimbic, anterior cingulate, and dorsal agranular insular cortex, whereas the shell receives projections predominantly from the infralimbic, ventral agranular insular, and piriform cortex (Zahm, 2000). Furthermore, and perhaps more importantly, these divisions innervate distinct areas; the core projects to the conventional basal ganglia circuitry including the ventral pallidum, globus pallidus, and the substantia nigra, whereas the shell projects to subcortical limbic structures such as the lateral hypothalamus and the ventral tegmental area (Zahm and Brog, 1992). These patterns of connectivities indicate that the two NAcc divisions may be involved in different aspects of the motivation-reward processes (Ikemoto and Panksepp, 1999) and the timing of the initiation of response patterns originating in the frontal corticostriatal loop systems (Groenewegen et al., 1999; Kelley et al., 2005).

The NAcc receives major dopaminergic inputs from the VTA. Importantly, the central dopaminergic systems, especially those in the NAcc that are crucial to the regulation of reward behavior, appear to be particularly vulnerable to perinatal insults. For example, maternal stress in late gestation (Berger et al., 2002; Diaz et al., 1998; Henry et al., 1995) or repeated periods of maternal separation during early postnatal development (Meaney et al., 2002) alter the pattern of DA receptor expression and decrease the dopamine transporter availability in the NAcc, later in life. Interestingly, Meaney and collaborators (2002) showed that postnatal maternal separation produces increased behavioral responses to stress and cocaine. In addition, there is evidence that prenatal and early postnatal stress results in decreased DA drive to the medial prefrontal cortex (Brake et al., 2000), a feature commonly associated with psychiatric disorders thought to have a neurodevelopmental basis (Lewis and Levitt, 2002).

The mechanisms through which stress alters NAcc function have not been fully investigated. On the premise that elevation of glucocorticoids is one of the most overt physiological manifestations of stress and that the brain is more sensitive to organization by glucocorticoids during early development, this initial study evaluated the potential impact of antenatal glucocorticoid administration upon NAcc structure and its dopaminergic innervation. Our results show that exposure of the fetus to dexamethasone (DEX) during late gestation leads to a reduction in both the volume and the number of cells in the $NAcc_{core}$ and $NAcc_{shell}$ of males, and volumes and number of neurons in the $NAcc_{shell}$ in females. These changes could be partly attributed to antenatal DEX-induced inhibition of cell proliferation in NAcc. Further, we report an association between fetal exposure to DEX and reduced numbers of tyrosine hydroxylase (TH)-positive cells in the adult VTA, paralleled by a markedly reduced density of dopaminergic inputs to the NAcc.

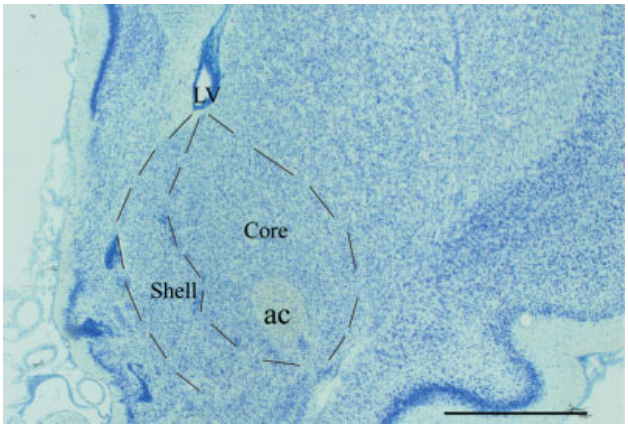

Fig. 1. Photomicrographs of Giemsa-stained glycolmethacrylate-embedded coronal sections of the NAcc shell and core divisions used for the cell counting with the SteroInvestigator software. LV, lateral ventricle; ac, anterior commissure. Scale bar = 200 μm.

MATERIALS AND METHODS

Animals and treatments

Adult pregnant Wistar rats (Charles River Laboratories, Barcelona, Spain) were individually housed under standard laboratory conditions (light/dark cycle of 12/12 h with lights on at 08:00; 22°C); food and water were provided ad libitum. Subcutaneous injections of dexamethasone (DEX, 0.1 mg/kg; $n = 4$) or saline (CONT, 1 ml/kg; $n = 4$) were administered on E18 and E19 days of pregnancy. Other groups of CONT- and DEX-treated mothers ($n = 3$) were given a single injection of bromodeoxyuridine (BrdU, 50 mg/kg, i.p.) on E18/19, to birthmark neurons in the offspring. On postnatal day 21, progeny were separated according to antenatal treatment and gender. All manipulations were done in accordance with local regulations (European Union Directive 86/609/EEC) and NIH guidelines on animal care and experimentation.

Tissue preparation

Groups of male and female rats born to mothers exposed to either saline or DEX (derived from four different litters) on E18 and E19 of pregnancy were deeply anesthetized and transcardially perfused with 4% paraformaldehyde (PFA) when 60 days old. Brains were removed and the cerebral hemispheres were separated by a longitudinal cut in the midsagittal plane. Blocks containing the NAcc were washed in tap water and dehydrated through a graded series of ethanol solutions, before being embedded in glycolmethacrylate (Tecnovit 7100, Heraeus Kulzer, Wehrheim, Germany). Microtome sections (30 μm) were placed on slides

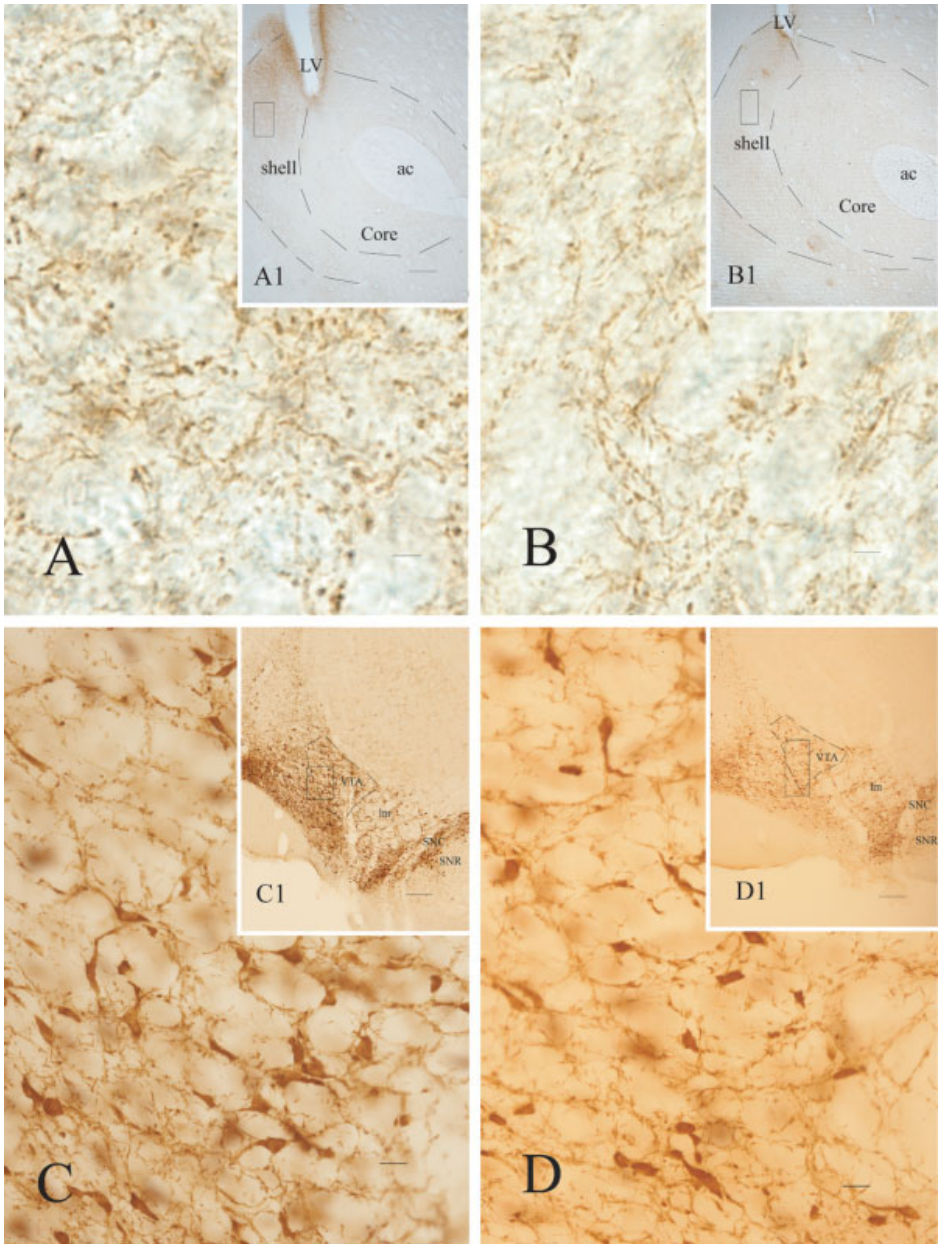

Fig. 2. Representative light microscopic photomicrographs of TH-positive immunocytochemistry in NAcc and VTA. Illustration of dopaminergic innervation of NAcc in control- (**A**) and DEX-treated (**B**) animals. Magnification of control- (**A1**) and DEX-treated (**B1**). Boxes represent the areas illustrated in (**A**) and (**B**), respectively. TH immunocytochemistry in control (**C,C1**) and DEX-treated (**D, D1**) in the VTA. LV, lateral ventricle; ac, anterior commissure. (A–D) – Scale bar = 200 μm and (A1–D1) – Scale bar = 5 μm.

coated with Entellan-new, before staining with Giemsa stain. The outline of the NAcc, including its core and shell areas (Zahm and Brog, 1992), was defined in each section using established landmarks (Fig. 1).

Another subset of male rats (derived from four different litters) was prepared for TH immunocytochemistry. Every 8th (30-μm thick) section, which included the NAcc and VTA, was treated with 3% H_2O_2 in PBS to eliminate endogenous peroxidase activity and blocked with 4% bovine serum albumin (BSA, Sigma) in PBS. Sections were then incubated overnight at 4°C in rabbit anti-TH serum (1:2000; Affinity Reagents, Exeter, UK), in blocking solution. Antigen visualization was carried out by sequentially incubating with biotinylated goat antirabbit antibody, ABC® (Vector, Burlingame, CA), and diaminobenzidine (DAB, Sigma) (Fig. 2).

Serial coronal cryosections, covering the entire length of the NAcc and VTA, were prepared from 3-day old pups (derived from three different litters) whose mothers had been treated with BrdU (Fig. 3). Every 2nd section from this series was mounted on poly-L-lysine-coated slides and stained for BrdU after fixation in 4% PFA (30 min), permeabilization (0.2% Triton X-100 in Tris buffer saline, 10 min), microwave treatment for antigen retrieval (20 min in 0.1 M citrate buffer), acidification with 2 M HCl, peroxidase and nonspecific blocking (3% H_2O_2 and 4% BSA, respectively). The BrdU antibody was a mouse monoclonal from Dako,

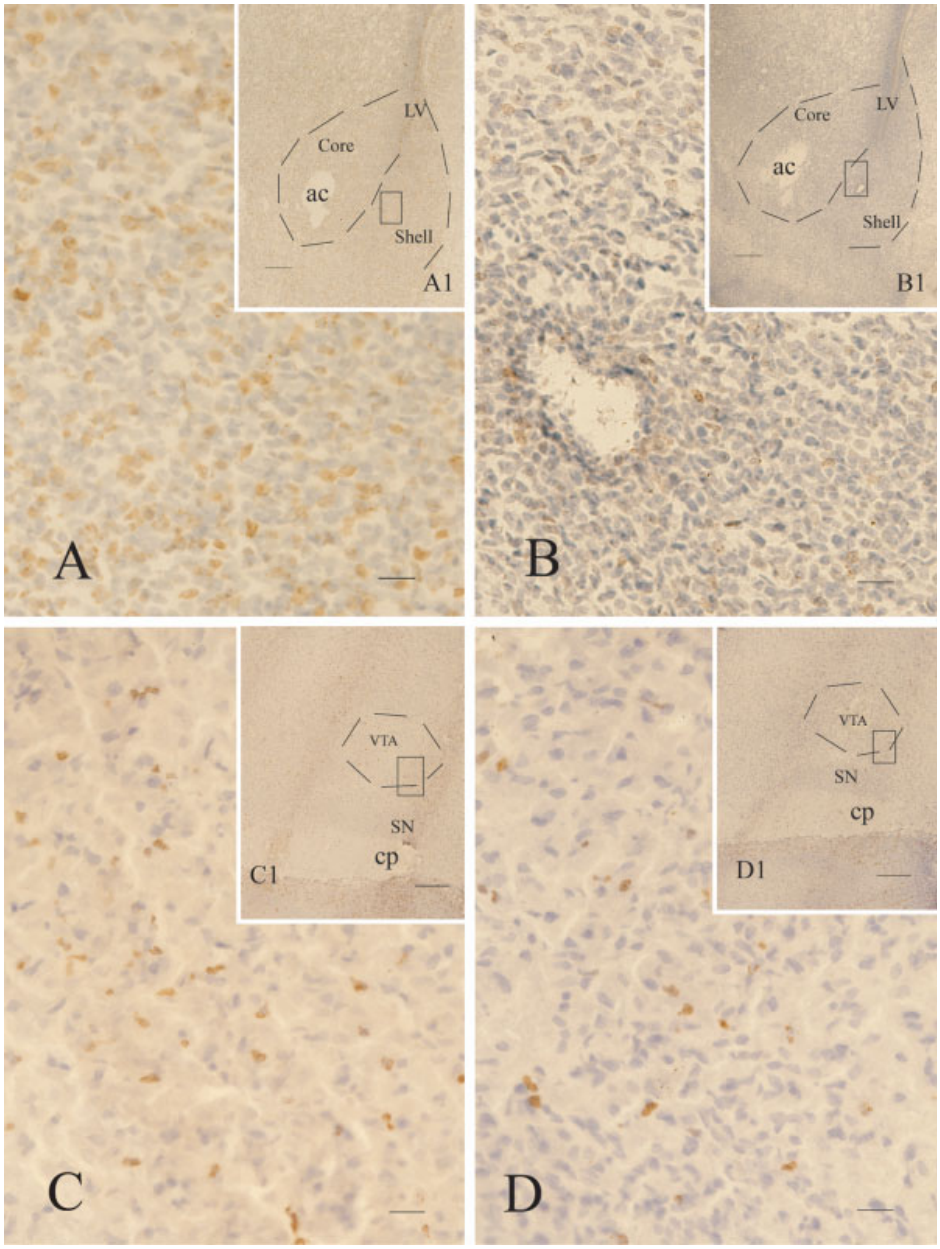

Fig. 3. Photomicrographs demonstrating BrdU immunoreactive cells in NAcc of controls (**A,A1**), VTA control (**C,C1**), DEX-treated (**B,B1**), and VTA DEX-treated (**D,D1**) in rats of PND3. (A–D) – Scale bar = 200 μm; (A1–D1) – Scale bar = 5 μm

(Glostrup, Denmark) (1:50). Antigen visualization was carried out using a universal detection system (BioGenex, San Ramon, CA) and DAB. Specimens were lightly counterstained with hematoxylin.

Stereology

Estimates of $NAcc_{core}$ and $NAcc_{shell}$ region volumes and cell numbers were obtained using StereoInvestigator® software (MicroBrightfield, VT) and a camera (DXC-390, Sony, Japan) attached to a motorized microscope (Axioplan 2, Carl Zeiss, Germany). Cavalieri's principle was used to assess the volume of each region. Every 8th section was used, and the cross-sectional area was estimated by point counting (final magnification ×112). We used a test point system in which the interpoint distance, at the tissue level, was 75 μm. The volume of the region of interest (ROI) was then calculated from the number of points that fell within its boundaries and the distance between the systematically sampled sections. Average cell numbers were estimated using the optical fractionator method as described elsewhere (West et al., 1991). Briefly, a grid of virtual 3D-boxes (30 μm × 30 μm × 15 μm) was superimposed on every 8th section of the ROI, spaced as for the volumetric estimation. An estimate of total number of cells was then derived from the number of cells falling inside the boxes, the volume of each box,

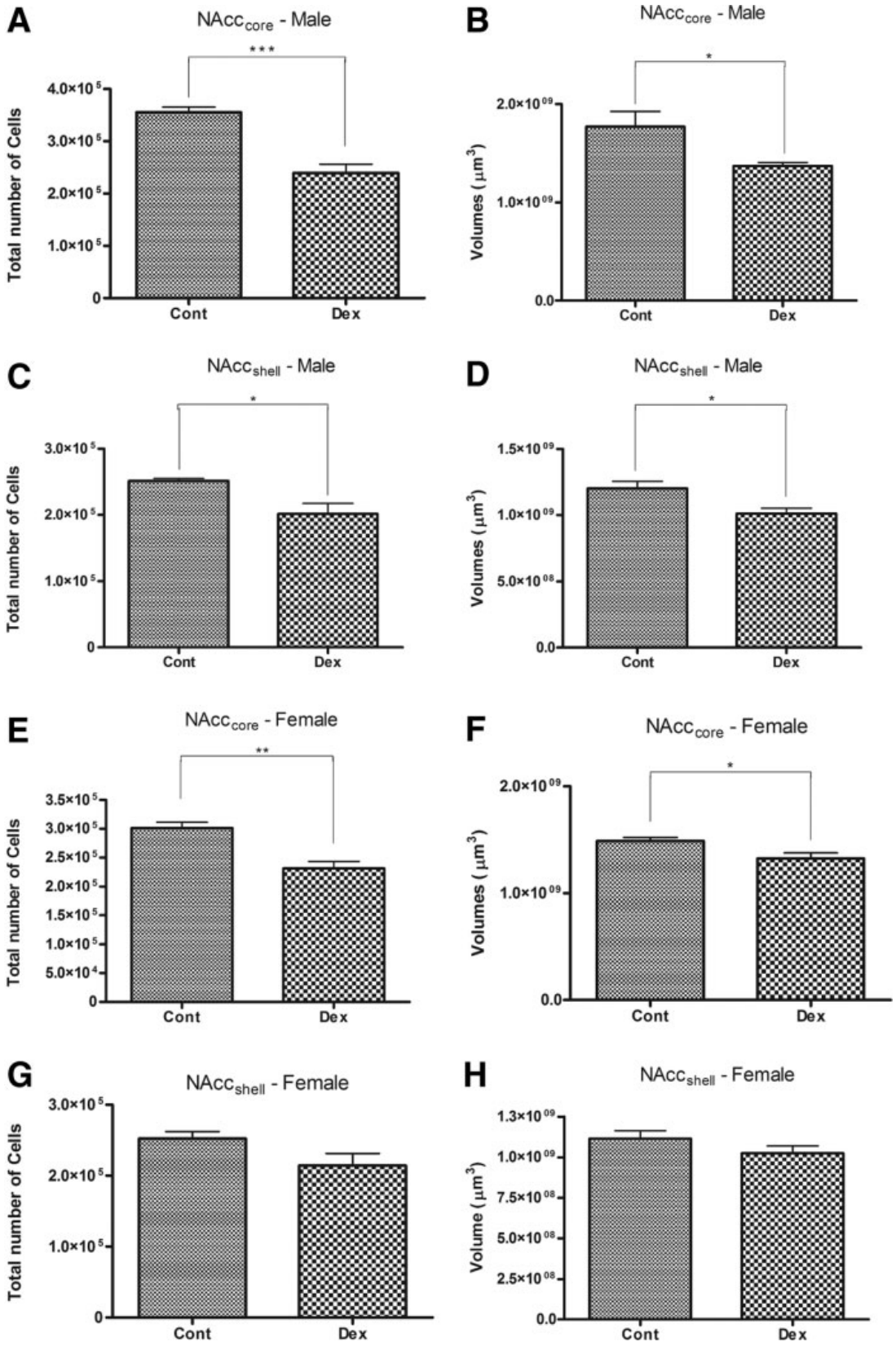

Fig. 4. Stereological data. Average volumes of NAcc regions in males (**A,C**) and females (**E,G**). Total number of neurons in NAcc divisons in males (**B,D**) and females (**F,H**). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

box spacing, and total number of boxes. Coefficients of error were automatically computed according to the formulas of Gundersen et al. (1999) for cell numbers and Gundersen et al. (1987) for volume estimations. Glial cells were not included in the estimations, and the discrimination between neuronal and glial cell body profiles was based on the criteria described by Ling et al. (1973) and Peinado et al. (1997).

The density of TH-positive fibers impinging upon the NAcc was estimated using a "staggered" cycloid test system (Baddeley et al., 1986; Emre et al., 1993; Issacs et al., 1993). The total number of intersections of the cycloid arcs with the stained fibers was obtained on randomly selected sections containing the core and shell regions. In addition, the number of TH-positive (VTA) and of BrdU-positive (NAcc and VTA) cells per unit area was estimated using StereoInvestigator software. To complement these measurements, a densitometric analysis of TH immunoreactivity in the NAcc (core and shell) was performed using NIH Image 1.52 software; the unit sampling area for these measurements was 1.0 mm^2, and measurements were made in three randomly-selected regions within core and shell of the NAcc.

Statistical analysis

Differences between groups were determined by a nonparametric procedure (Mann–Whitney) that does not assume normality or equal variance. For statistical analysis, the "*n*" of each experiment group is the number of litters from which individuals were derived. The results are expressed as group means ± standard error. Differences were considered to be statistically significant when $P < 0.05$.

RESULTS

Sustained effects of antenatal DEX on NAcc cytoarchitecture

The male progeny of DEX-treated dams displayed reduced volume ($P = 0.03$) and cell number ($P = 0.001$) in the $NAcc_{core}$ (Figs. 4A and 4B). $NAcc_{core}$ male controls: total number of cells = 352,000 (SEM = 8155, $n = 4$), volume = 1.75 mm^3 (SEM = 0.13, $n = 4$); male DEX: total number of cells = 235,600 (SEM = 19,050, $n = 4$), volume = 1.35 mm^3 (SEM = 0.03, $n = 4$). Similar observations, albeit of smaller magnitude, were made in their female counterparts (volume: $P = 0.001$; cell number: $P = 0.004$; Figs. 4E and 4F). $NAcc_{core}$ female controls: total number of cells = 306,400 (SEM = 11,720, $n = 4$), volume = 1.49 mm^3 (SEM = 0.03, $n = 4$); female DEX: total number of cells = 231,500 (SEM = 12.160, $n = 4$), volume = 1.27 mm^3 (SEM = 0.048, $n = 4$).

Prenatal DEX also significantly reduced the volume ($P = 0.04$) and number of cells ($P = 0.02$) in the $NAcc_{shell}$ of males (Figs. 4C and 4D). $NAcc_{shell}$ male controls: total number of cells = 252,000 (SEM = 4121, $n = 4$), volume = 0.12 mm^3 (SEM = 0.055, $n = 4$); male DEX: total number of cells = 199,600 (SEM = 17,290, $n = 4$), volume = 0.9 mm^3 (SEM = 0.03, $n = 4$). In contrast, there was no significant effect of antenatal DEX treatment in the shell region of the NAcc in females (volume: $P = 0.7$; cell number: $P = 0.4$) (Figs. 4G and 4H). $NAcc_{shell}$ female controls: total number of cells = 234,700 (SEM = 1649, $n = 4$), volume = 1.1 mm^3 (SEM = 0.03, $n = 4$); female

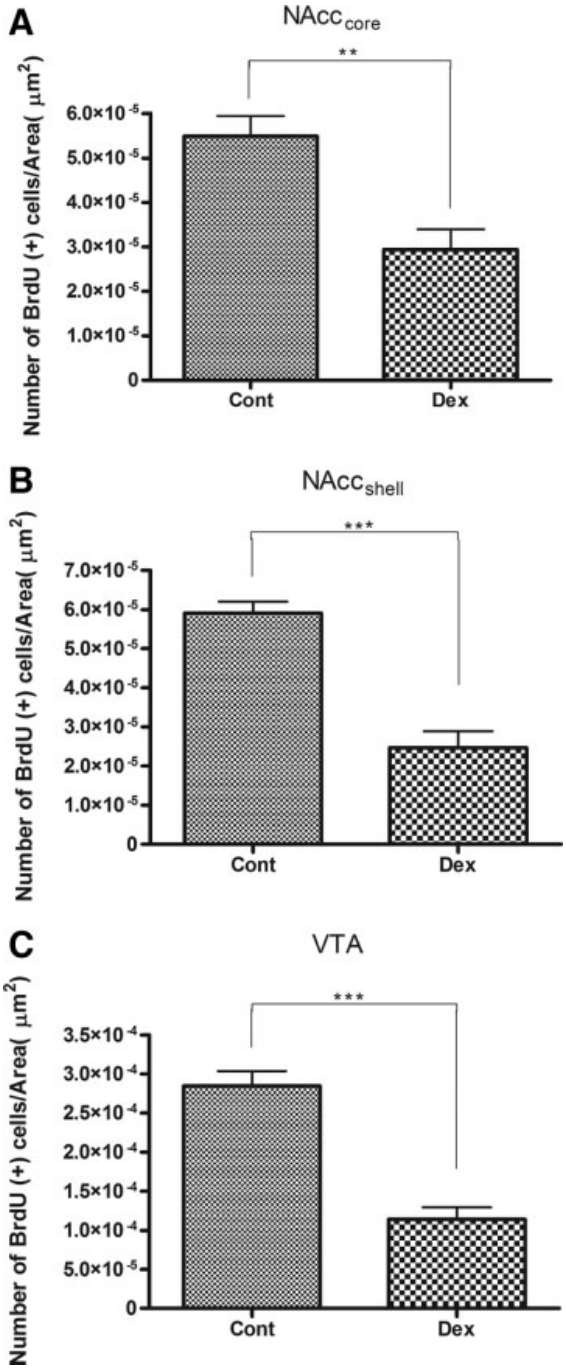

Fig. 5. Number of BrdU-positive cells in NAcc regions (**A,B**) and VTAs (**C**) in PND3. $^{**}P < 0.01$; $^{***}P < 0.001$.

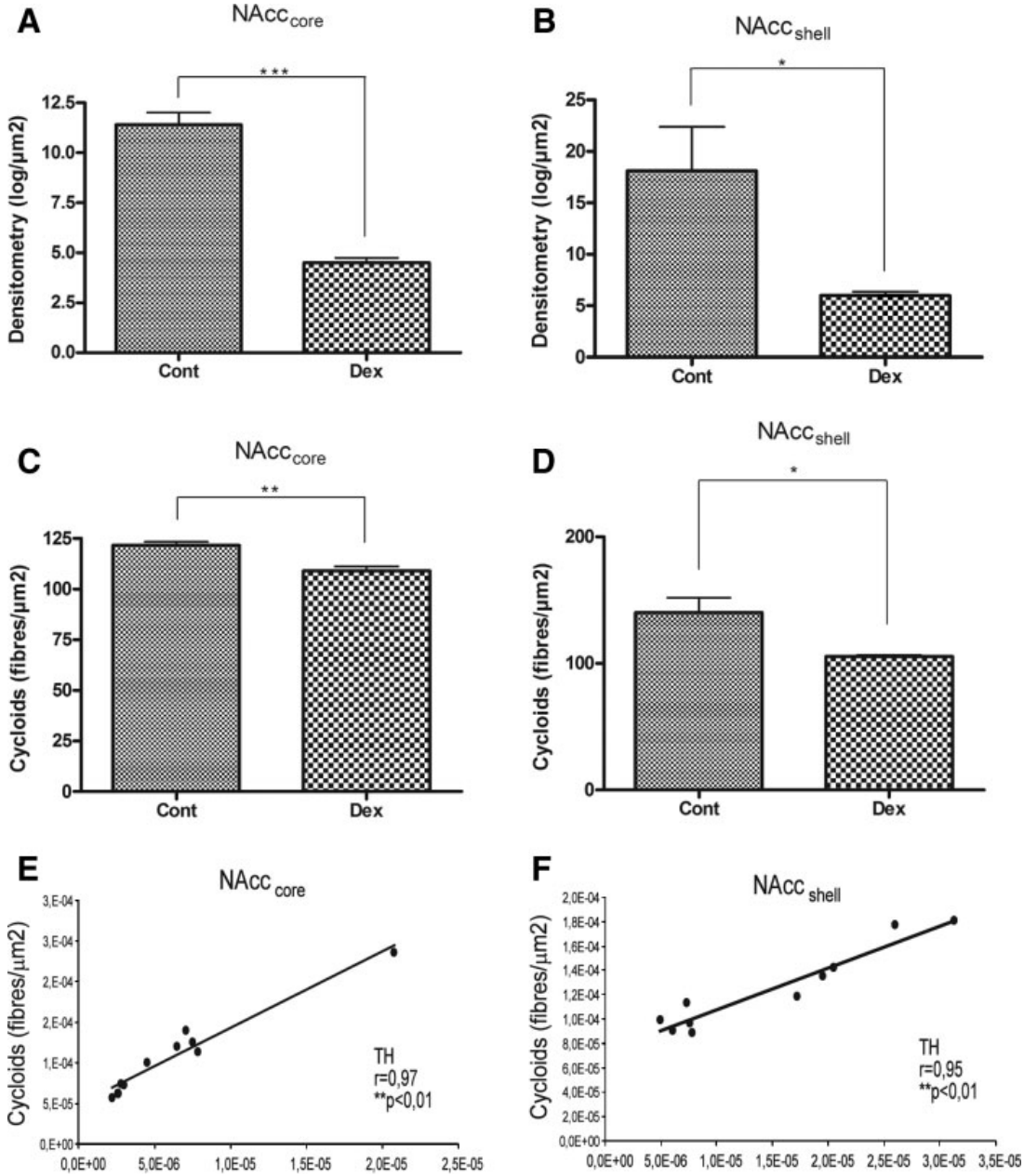

Fig. 6. Decreased TH content in the NAcc divisions of the progeny, after treatment with prenatal DEX. Densitometric (**A,B**) and stereological (**C,D**) data in core and shell, respectively. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Pearson correlation of both densitometric and stereologic quantifications of TH immunoreactive fibers in NAcc core (**E**) and shell (**F**).

DEX: total number of cells = 214,200 (SEM = 16,780, $n = 4$), volume = 1.02 mm^3 (SEM = 0.04, $n = 4$).

Inhibition of cell proliferation in the NAcc and VTA

The total number of cells in a given tissue is determined by the dynamic balance in cell proliferation and cell death (cell turnover). In view of the reduced number of cells in the NAcc core and shell of male progeny of DEX-treated mothers, as well as the known inhibitory effects of stress and glucocorticoids on neurogenesis, in this first study, we examined the possible contribution of altered cell proliferation on total NAcc cell numbers in male animals. As shown in Figures 5A and 5B, exposure to antenatal DEX resulted in a significant inhibition of BrdU incorporation (assessed at postnatal day 3) in the NAcc core ($P = 0.004$) and shell ($P = 0.0003$) regions, as well as in the VTA ($P = 0.0001$) (Fig. 5C).

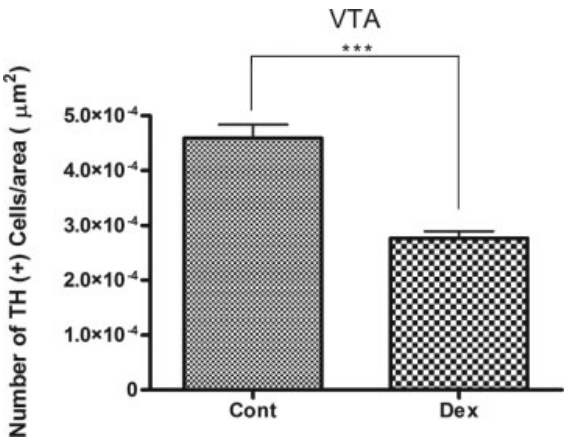

Fig. 7. TH-positive cells per surface unit of the VTA. ***$P < 0.0001$.

Reduced dopaminergic innervation of the NAcc after prenatal exposure to DEX

Densitometric and stereological measurements in the two divisions of the NAcc revealed significantly reduced densities of TH-positive fibers in the progeny of DEX-treated dams (by densitometry: core, $P = 0.04$ (Fig. 6A), and shell, $P = 0.001$ (Fig. 6B); by stereology: core, $P = 0.03$, (Fig. 6C) and shell, $P = 0.003$ (Fig. 6D). The correlation coefficient (Pearson analysis) between the two methods of estimation for core was $r = 0.97$ and $P < 0.01$ (Fig. 6E); shell: $r = 0.95$ and $P < 0.01$ (Fig. 6F).

Supplementing the earlier findings, we observed that the number of TH-positive cells per surface unit of the VTA was significantly reduced in animals that had received DEX during prenatal life ($P = 0.0003$) (Fig. 7).

DISCUSSION

Stressful experience during uterine or early postnatal life is thought to predispose individuals to addictive habits, as well as to increased risk for developing mental disorders such as schizophrenia and major depression (Hougaard et al., 2005; McClure et al., 2004). Motivation and the ability to respond to rewarding stimuli are key elements in all of these conditions. Work over the last two decades has identified the mesolimbic "reward pathway," of which the NAcc and VTA are crucial components (Wise, 2004a,b). Importantly, the VTA projects dopaminergic terminals to the NAcc (Jonjen-Relo et al., 1994). Since the NAcc plays a significant role in the timing of the initiation of response patterns originating in the frontal corticostriatal loop systems (Groenewegen et al., 1999), its implication in drug addiction and anhedonia (Di Chiara, 2002; Salamone et al., 2003) is not surprising.

Increased CS secretion is a key feature of the response to stress. CSs are small, lipid-soluble molecules; these characteristics allow them easy access to the brain, where they elicit both rapid and long-lasting changes in neural activity and behavior. Numerous studies from our own and other laboratories have demonstrated that GC can induce structural changes in various brain regions including the prefrontal cortex (Brown et al., 2005; Cerqueira et al., 2005), hippocampus (Donohue et al., 2006; Sousa et al., 1999, 2000), and striatum (Copeland et al., 2005; Haynes et al., 2001).

Recently, McClure et al. (2004) reported a reduction in the volume and number of neurons in the NAcc of rats born to mothers that had received an injection of saline during late pregnancy; they putatively attributed these structural changes to injection stress-induced CS secretion. The results of the present study largely confirm the supposition by McClure et al. (2004). We show that the adult offspring of rat dams that had been exposed to the synthetic CS DEX during the late stage of pregnancy have significantly decreased $NAcc_{core}$ volumes and total neuronal numbers. Similar observations were made in the $NAcc_{shell}$ of the male, but not female, progeny of DEX-treated mothers. While the significance of this sex difference is not known at present, it is interesting to note that males show greater susceptibility to early life events (McClure et al., 2004; Simon and Volicer, 1976) and that, in humans and animals, the two sexes display differences in their propensity to develop addictive behavior, and mood and other affective disorders (Simon and Volicer, 1976). Further, the two sexes differ significantly in their basal CS secretory profiles and endocrine responses to stress (Patchev et al., 1997), as well as in their hormonal and behavioral responses to prenatal stress (Bowman et al., 2004). Our finding that CS influences the structure of the $NAcc_{shell}$ (but not $NAcc_{core}$) in a sex-specific fashion calls for the establishment of the relationship between sex, stress, and the regulation of specific components of the motivation-reward circuit. Current views hold that the $NAcc_{shell}$ is responsible for anticipatory responses (Chang et al., 1994), while the $NAcc_{core}$ participates in the timing of the initiation of response patterns originating in the frontal corticostriatal loop systems; it will be interesting to know whether these processes are differentially regulated between the sexes and to what extent their different susceptibilities to antenatal CS (and presumably stress) translates into sex differences in behavior.

The changes in NAcc volume and neuronal numbers in animals exposed to antenatal CS were accompanied by significant reductions in the dopaminergic innervation of the NAcc by the VTA (confirming a recent report by McArthur et al., 2005), as well as by reduced numbers of dopaminergic cell bodies in the VTA (not observed by McArthur et al., 2005). Interestingly, these

changes appear to result from antenatal DEX-induced alterations in cell proliferation: our results record, for the first time, that CS can significantly reduce postnatal neurogenesis in both, the VTA and NAcc. The inhibitory actions of CS on neuronal proliferation in the postnatal brain, especially in the hippocampus (Gould et al., 1991), have been extensively described, but their ability to interfere with neurogenesis during prenatal life have been recognized only recently (Lemaire et al., 2000). CS receptors are expressed in many areas of the rat brain, including the basal ganglia striatum and brainstem nuclei, from embryonic day 15.5 (Diaz et al., 1998); since the NAcc and VTA undergo considerable structural organization during late gestation (Hynes and Rosenthal, 1999), our antenatal CS paradigm on E18/19 is likely to have had a major impact on this process.

The NAcc seems to be one of the most important brain centers in determining drug-addiction behavior. When stimulated by DA, neurons in NAcc produce feelings of pleasure and satisfaction; in normal conditions, such response keeps the subject focused on basic biological goals. However, genetic as well as natural and pharmacological interventions can modulate dopaminergic drive upon the NAcc. The decrease in basal dopaminergic in the NAcc after prenatal DEX treatment (associated with an impoverishment structural complexity in this nucleus) may determine an increase susceptibility to drug-seeking behavior. Thus, the results of the present study lend support to the view that early exposure to stress or CS might ''program'' the mechanisms and neural substrates governing reward-seeking behavior throughout the lifespan (Marinelli and Piazza, 2002; Piazza et al., 1998).

Importantly, the implications of the present results are plausibly also relevant to our understanding of the biological basis of schizophrenia, since there are reports that cell losses contribute to volumetric reductions in the NAcc of schizophrenic patients (Pakkenberg, 1990). Moreover, CS released during stress might contribute to the neurodevelopmental anomalies that underlie schizophrenia (Boksa and El-Khodor, 2003); in support to this hypothesis, Koenig et al. (2005) have shown that two parameters used for the validation of animal models of schizophrenia—diminution of the prepulse inhibition of the acoustic startle response and disruption of auditory sensory gating—are modulated by prenatal stress. In addition, our findings that antenatal CS exposure leads to decreased DA input to the NAcc and reduced numbers of TH-positive cells in the adult VTA show that CS may be important determinants of mesolimbic function in the adult. Lastly, our observations that antenatal exposure to CS can have enduring effects on the cytoarchitecture and neurochemistry of the VTA and NAcc are important in the light of the common use of CS in the prevention of preterm birth in women.

Synapse DOI 10.1002/syn

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Appendix 2

Rodrigues AJ, Leão P, Pêgo JM, Cardona D, Carvalho MM, **Oliveira M**, Costa B, Carvalho AF, Morgado P, Araújo D, Palha JA, Almeida OFX, Sousa N

Mechanisms of initiation and reversal of drug-seeking behavior induced by prenatal exposure to glucocorticoids

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ORIGINAL ARTICLE

Mechanisms of initiation and reversal of drug-seeking behavior induced by prenatal exposure to glucocorticoids

AJ Rodrigues[1,2,4], P Leão[1,2,4], JM Pêgo[1,2], D Cardona[1,2], MM Carvalho[1,2], M Oliveira[1,2], BM Costa[1,2], AF Carvalho[1,2], P Morgado[1,2], D Araújo[1,2], JA Palha[1,2], OFX Almeida[3] and N Sousa[1,2]

[1]*Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga, Portugal;* [2]*ICVS/3B's–PT Government Associate Laboratory, Braga/Guimarães, Portugal and* [3]*Neuroadaptations Group, Max Planck Institute of Psychiatry, Munich, Germany*

Stress and exposure to glucocorticoids (GC) during early life render individuals vulnerable to brain disorders by inducing structural and chemical alterations in specific neural substrates. Here we show that adult rats that had been exposed to *in utero* GCs (iuGC) display increased preference for opiates and ethanol, and are more responsive to the psychostimulatory actions of morphine. These animals presented prominent changes in the nucleus accumbens (NAcc), a key component of the mesolimbic reward circuitry; specifically, cell numbers and dopamine (DA) levels were significantly reduced, whereas *DA receptor 2* (*Drd2*) mRNA expression levels were markedly upregulated in the NAcc. Interestingly, repeated morphine exposure significantly downregulated *Drd2* expression in iuGC-exposed animals, in parallel with increased DNA methylation of the *Drd2* gene. Administration of a therapeutic dose of L-dopa reverted the hypodopaminergic state in the NAcc of iuGC animals, normalized *Drd2* expression and prevented morphine-induced hypermethylation of the *Drd2* promoter. In addition, L-dopa treatment promoted dendritic and synaptic plasticity in the NAcc and, importantly, reversed drug-seeking behavior. These results reveal a new mechanism through which drug-seeking behaviors may emerge and suggest that a brief and simple pharmacological intervention can restrain these behaviors in vulnerable individuals.
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Introduction

Stressful events during critical developmental periods have long been considered as etiological factors in psychiatric disorders such as schizophrenia, depression and drug-seeking behavior.[1–4] The programming effects of stress are most likely mediated by endogenous glucocorticoids (GC), whose ability to produce structural re-organization and dysfunction of the neural substrates that underpin these stress-related pathologies are well known.[1,5–7] Although administration of prenatal GC does not mimic prenatal stress, synthetic GC such as dexamethasone (DEX) are widely used in obstetrics, for example, to ensure fetal lung maturation during late pregnancy in humans.[8] DEX is not biodegraded in the same way as its naturally occurring congeners, and crosses the maternal-placental barrier to a greater extent than endogenous GC;[9,10] it can thus pose additional risk for the developing brain.

We previously demonstrated that fetal exposure to GC leads to hyper-emotionality in adulthood.[11] In addition, we showed that prenatal DEX/GC targets the mesolimbic dopaminergic system;[12] this system comprises projections from the ventral tegmental area (VTA) to the nucleus accumbens (NAcc) and is strongly implicated in motivational and reward aspects of addictive behaviors.[13–15] Specifically, the NAcc of adult rats exposed to GC *in utero* (iuGC) display reduced neuronal numbers and fewer dopamine (DA) inputs from the VTA.[12] Further, early life stress is known to influence DA receptor expression in the adult NAcc[16,17] and changes associated with increased behavioral responses to stress and cocaine.[1,4,18,19] Together, these observations suggest that prenatal exposure to elevated levels of GC can program the mesolimbic circuit. In the present study, a multimodal analysis was used to further define the molecular neurobiological mechanisms that underlie the initiation and reversibility of drug-seeking behavior by prenatal exposure to GC.

Correspondence: Dr N Sousa, Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal.
E-mail: njcsousa@ecsaude.uminho.pt
[4]These authors contributed equally to this work.
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Materials and methods

Animals and behavioral tests
Pregnant Wistar rats were individually housed under standard laboratory conditions (light/dark cycle of 12/12 h with lights on at 08:00 h; 22 °C); food and water were provided *ad libitum*. Subcutaneous (s.c.) injections of DEX at 1 mg kg^{-1} (DEX; iuGC animals) or saline (control) were administered on gestation days 18 and 19. All manipulations were done in accordance with the local regulations (European Union Directive 2010/63/EU) and NIH guidelines on animal care and experimentation.

Male offspring ($n \geqslant 8$) derived from four different litters were subjected to behavioral tests when they were 3–4 months old.

Open field
Locomotor behavior was investigated using the open-field test. Briefly, rats were placed in the center of an arena (MedAssociates, St Albans, VT, USA) and their ambulation was monitored online over a period of 15 min. Total distances traveled were used as indicators of locomotor activity. Animals were injected with saline or morphine and tested 30 min after injection.

Conditioned place preference (CPP)
The place preference apparatus consisted of two compartments with different patterns on floors and walls, separated by a neutral area (MedAssociates). Animals were placed in the central neutral area and allowed to explore both compartments, allowing definition of the preferred compartment (day 1). During the conditioning phase (day 2–4), rats were confined to the pre-test preferred compartment for 20 min after saline injection (1 ml kg^{-1}, s.c.) and, after a 6-h gap, to the other compartment for 20 min after injection of morphine (10 mg kg^{-1}, s.c.). CPP was assessed on day 5 (20 min) when all compartments were accessible to the animal. Results are expressed as the difference of time spent in the drug-paired to saline-paired side.

Ethanol consumption
The two-bottle choice protocol was carried out for 15 days as described previously.[20] Briefly, after 3 days of taste habituation (one bottle with 10% ethanol and other with 5% sucrose), rats were offered both bottles. Each bottle was weighted daily; bottle positions were changed every day to control for position preference. Corrections were made for daily evaporation and spillage.

Cross-fostering and maternal behavior
For cross-fostering experiments, litters from five control and five DEX-treated mothers were exchanged on postnatal day 1. Maternal behavior was assessed every second day, over a period of 30 min. Both, pup-directed (nursing, non-nutritive contact, licking and nest building) and self-directed (self-grooming, resting, vertical activity and carrying) behaviors were registered.

Drugs
Morphine hydrochloride (Labesfal Pharmaceutical, Campo de Besteiros, Portugal) was administered s.c. at a dose of 10 mg kg^{-1}; sesame oil was used as the vehicle. L-dopa/carbidopa (Sinemet, Merck, NJ, USA) at a dose of 36.0/9.0 mg/kg (in water) was administered daily by oral gavage.

Tyrosine hydroxylase (TH) immunohistochemistry
Animals were deeply anesthetized and transcardially perfused with 4% paraformaldehyde. Cerebral hemispheres were separated by a longitudinal cut in the midsagittal plane. Sections of 30 μm were treated with 3% H_2O_2 and blocked with 4% bovine serum albumin in phosphate-buffered saline. Sections were then incubated overnight at 4 °C with rabbit anti-TH serum (1:2000; Affinity Reagents, CO, USA). Antigen visualization was carried out by sequentially incubating with biotinylated goat anti-rabbit antibody, ABC1 (Vector, Burlingame, CA, USA) and diaminobenzidine (DAB, Sigma). The density of TH-positive fibers impinging upon the NAcc was estimated as previously described.[12]

Structural analysis
Rats were transcardially perfused with 0.9% saline under deep pentobarbital anesthesia and processed as described previously.[21] Briefly, brains were removed and immersed in Golgi–Cox solution[22] for 14 days; brains were then transferred to a 30% sucrose solution (7 days), before being cut on a vibratome. Coronal sections (200 μm thick) were collected and blotted dry onto cleaned, gelatin-coated microscope slides. They were subsequently alkalinized in 18.7% ammonia, developed in Dektol (Kodak, Rochester, NY, USA), fixed in Kodak Rapid Fix (prepared as per package instructions with solution B omitted), dehydrated through a graded series of ethanols, cleared in xylene, mounted and coverslipped. For each selected neuron, all branches of the dendritic tree were reconstructed at ×600 magnification, using a motorized microscope with oil objectives (Axioplan 2, Carl Zeiss, Thornwood, NY, USA) that was attached to a camera (DXC-390, Sony, Tokyo, Japan) and Neurolucida software (Microbrightfield, Williston, VT, USA). A 3D analysis of the reconstructed neurons was performed using NeuroExplorer software (Microbrightfield). Twenty neurons were studied in each animal, and results from the same animal were averaged. To assess differences in the arrangement of dendritic material, a 3D version of a Sholl analysis[23,24] was performed. For this, we counted the number of intersections of dendrites with concentric spheres positioned at radial intervals of 20 μm; in addition, we also measured dendritic tree lengths located between two consecutive spheres. The method for sampling dendritic branches for spine density was designed as follows: only branches that (1) were either parallel or at acute angles to the coronal surface of the section and (2) did not show overlap with other branches that would obscure visualization of spines

were considered. Because treatment-induced changes in the apical dendritic branches varied with distance to soma, segments were randomly selected in the proximal parts of the tree; selection of basal dendrite was done at radial distances between 50 and 100 μm. To assess treatment-induced changes in spine morphology, spines in the selected segments were classified according to Harris *et al.*[25] in mushroom, thin, wide and ramified categories. Thin spines were considered immature, whereas the other spine types were considered to be mature spines.

Macrodissection
Animals were anesthetized, decapitated, and heads were immediately snap-frozen in liquid nitrogen. Brain areas of interest were rapidly dissected on ice under a stereomicroscope, observing anatomical landmarks. Samples were snap-frozen (dry ice) and stored at −80 °C until use.

Neurochemical evaluation
Levels of catecholamines were assayed by high-performance liquid chromatography, combined with electrochemical detection (HPLC/EC) using a Gilson instrument (Gilson, Middleton, WI, USA), fitted with an analytical column (Supleco Supelcosil LC-18 3 μM, Bellefonte, PA, USA; flow rate: 1.0 ml min^{-1}). Samples were stored overnight in 0.2 N perchloric acid at −20 °C, sonicated (5 min on ice) and centrifuged at 5000 *g*. The resulting supernatant was filtered through a Spin-X HPLC column (Costar, Lowell, MA, USA) to remove debris and 150 μl aliquots were injected into the HPLC system, using a mobile phase of 0.7 M aqueous potassium phosphate (pH 3.0) in 10% methanol, 1-heptanesulfonic acid (222 mg l^{-1}) and Na-EDTA (40 mg l^{-1}). A standard curve using known concentrations of all catecholamines was run each day.

Molecular analysis
For real-time PCR analysis, total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA) and DNase treated (Fermentas, Burlington, Canada) following recommended protocols. Two μg of RNA was converted into cDNA using the iSCRIPT kit (Biorad, Hercules, CA, USA). Reverse transcription PCR was performed using Quantitec SyberGreen (Qiagen, Venlo, The Netherlands) and the Biorad q-PCR CFX96 apparatus. *Hprt* was used as a housekeeping gene. Relative quantification was used to determine fold changes (control vs iuGC), using the ΔΔCT method. Primer sequences are shown in Supplementary Table 1.

For western blotting procedures, ice-cold lysis buffer (50 mM Tris-HCl pH 7.4, 50 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, complete protease inhibitors (Roche, Basel, Switzerland)) was added to each frozen area. After disruption of the tissue using a 23G needle, 0.1% SDS and 1% Triton X-100 was added to each sample. After incubation on ice for 1 h, samples were centrifuged at 13 000 r.p.m. for 10 min at 4 °C; the supernatant was quantified using the Bradford method. Forty μg of total protein was loaded into SDS-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes. After incubation with the primary antibodies: rabbit anti-Dopamine receptor D1 (1:2500, ab20066, Abcam, Cambridge, UK), rabbit anti-Dopamine receptor D2 (1:2000, ab21218, Abcam) and mouse anti-alpha-tubulin (1:200, DSHB, Iowa, USA); the secondary antibodies were incubated at a 1:10 000 dilution (Santa Cruz Biotechnologies, Santa Cruz, CA, USA). Detection was done using ECL kit (Pierce, Rockford, IL, USA). Band quantification was performed using ImageJ (http://rsbweb.nih.gov/ij/) as advised by the software manufacturers, using α-tubulin as the loading control. At least six animals per group were analyzed.

For epigenetic analysis, four animals per group were analyzed. Genomic DNA of 2 μg were bisulfite-converted (EZDNA Methylation Kit, Zymo Research, Irvine, CA, USA) and amplified with primers CpG-Drd2_F and CpG-Drd2_R (designed using Methprimer), using AmplitaQ Gold (Applied Biosystems, Carlsbad, CA, USA). Bands were purified using innuPREP Gel extraction kit (Analytik Jena, Jena, Germany). After elution, 2 μl of product were used in a TOPO cloning reaction (Invitrogen) following recommended procedures. XL1-blue competent cells were transformed with the TOPO reaction and plated onto LB–50 μg ml^{-1} kanamycin plates, suplemented with X-GAL (5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside). A total of 10 clones were isolated per animal; plasmid DNA was purified using innuPREP Plasmid Mini Kit. Plasmids were sequenced using standard M13 primers.

Results

In utero *GC exposure triggers increased drug-seeking behavior in adulthood*
To test the hypothesis that prenatal GC exposure would increase drug preference, we compared all experimental groups in a CPP paradigm. As compared with controls, iuGC-treated animals developed a stronger preference for morphine, spending more time in the compartment previously associated with morphine reward (Figure 1a; $t = 4.623$, $P = 0.0036$). Whereas control and iuGC animals did not differ in their intake of sucrose solution (Supplementary Figure S1), iuGC animals demonstrated an approximately two-fold greater preference than controls for ethanol in a two-bottle free-choice paradigm over a period of 2 weeks (Figure 1b; $t = 3.523$, $P = 0.0048$). As locomotor activity is considered to predict susceptibility to drug abuse,[1,26] it was interesting to note that morphine stimulated locomotor activity (open-field arena) to a greater extent in iuGC animals than in controls (~160% vs ~35%; $F_{(3,15)} = 67.94$, $P < 0.0001$; Figure 1c). To exclude the potentially confounding effects of inadequate maternal care, itself a suspected etiological factor in stress-related psychiatric disorders,[27–29] we analyzed the maternal behavior of control and GC-treated dams, and also performed a

cross-fostering experiment. Neither self- nor pup-directed behaviors were significantly influenced by GC treatment (Supplementary Figure S2). Identical behaviors were observed when iuGC offspring raised by natural and fostered mothers were compared in the CPP (Figure 1a; $t=6.877$, $P<0.0001$) or ethanol consumption (Figure 1b; $t=12.58$, $P<0.0001$) tests. Although the hypolocomotor profile observed in non-fostered iuGC animals in the open field test was not seen in cross-fostered iuGC rats (Figure 1c), morphine elicited a hyperlocomotor response in both cross-fostered and non-fostered iuGC animals as compared with control rats raised by foster mothers (Figure 1c; $t=2.737$, $P=0.021$). Collectively, these findings indicate that exposure to prenatal GC increases vulnerability to drug-seeking behavior.

Morphological and neurochemical changes in the NAcc after in utero *GC exposure*

Increased sensitivity to the psychomotor-stimulatory actions of drugs such as morphine reflects increased DA release into the NAcc.[1,26] Furthermore, the dopaminergic system seems particularly sensitive to the effects of GCs.[5,12,30] Thus, we next assessed the impact of prenatal GC upon the number of TH-positive fibers, DA and DA metabolite levels, as well as DA turnover in the NAcc (Figure 2). The number of TH-positive fibers in both the core and shell divisions of the NAcc were significantly reduced in iuGC animals (Figure 2a, shell: $t=2.827$, $P=0.022$; Figure; core: $t=10.48$, $P<0.0001$; Supplementary Figure S3), in parallel with markedly reduced NAcc levels of DA ($t=2.567$, $P=0.0247$) and the DA metabolite 3,4-dihydroxyphenylacetic acid (DOPAC; $t=2.362$, $P=0.0376$; Figure 2c); interestingly, the levels of norepinephrine and epinephrine, two other catecholamine transmitters whose synthesis indirectly depends on TH, as well as of the unrelated monoamine serotonin (5-HT), were not affected by prenatal GC exposure. Importantly, besides the reduced availability of DA in the NAcc, iuGC-treated animals also displayed increased DA turnover (Figure 2d; $t=2.835$, $P=0.0196$). Moreover, as no remarkable neurochemical changes were observed in the VTA or other DA projection fields (prefrontal cortex, hippocampus; data not shown), the

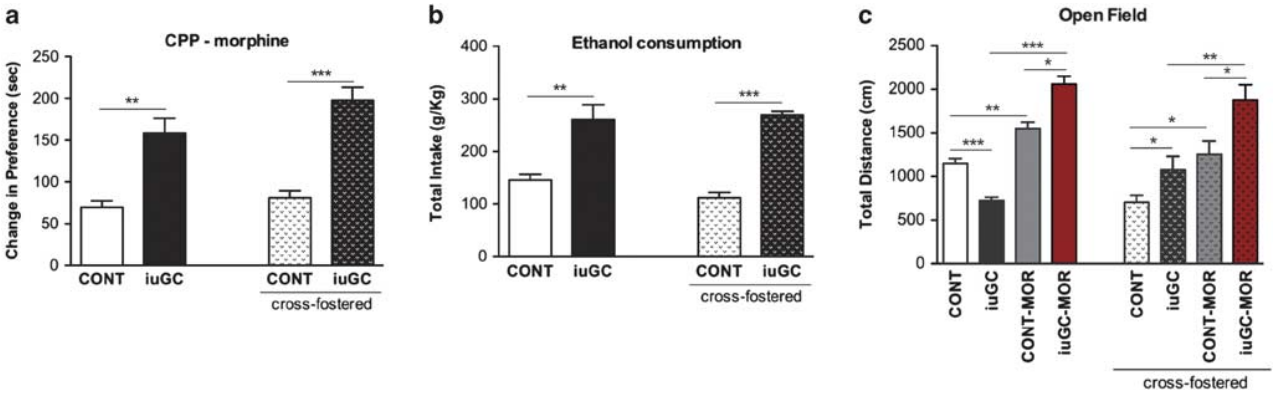
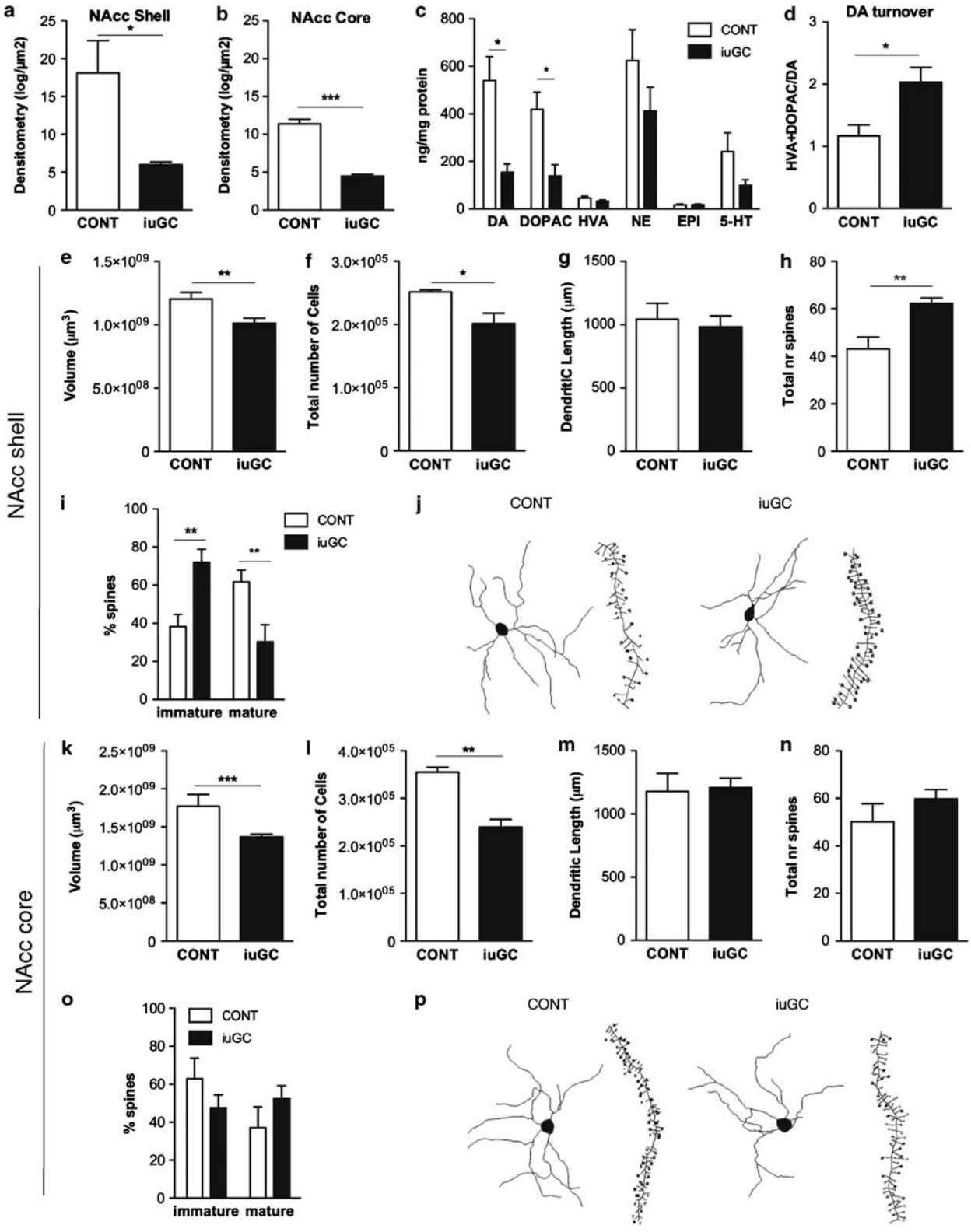


Figure 1 Prenatal *in utero* glucocorticoid (iuGC) exposure enhances drug-seeking behaviors. (**a**) In the contingent conditioned place preference paradigm (CPP), iuGC animals spend significantly more time in the morphine-associated compartment than controls. (**b**) In the non-contingent two-bottle preference paradigm, total ethanol consumption was higher in iuGC animals than in controls. Similar results were obtained for cross-fostered animals in both paradigms. (**c**) Locomotor activity was assessed in the open field. Although in basal conditions, iuGC animals presented reduced locomotor activity, after morphine administration (MOR), iuGC rats displayed increased locomotor activity when compared with controls. Cross-fostered iuGC-animals no longer present the basal hypolocomotor phenotype, but after MOR, they still presented increased locomotor activity. Data is presented as mean ± s.e.m. CONT, controls; MOR, morphine (10 mg kg^{-1}) s.c. injection. $^{*}P<0.05$, $^{**}P<0.01$,*** $P<0.001$.

Figure 2 Prenatal glucocorticoid (GC) reorganizes dopaminergic innervation and dendritic structure in the nucleus accumbens (NAcc). *In utero* GC-exposed (iuGC) animals presented reduced tyrosine hydroxylase (TH)-positive fibers in the shell (**a**) and core (**b**) subdivisions of the NAcc when adults. (**c**) High-performance liquid chromatography (HPLC) measurements confirmed reduced levels of dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanilic acid (HVA) in the NAcc of iuGC animals in comparison with controls in parallel with increased turnover of DA in this brain region (**d**). Stereological assessment revealed a volumetric atrophy (**e**) in the NAcc shell in iuGC animals together with reduced number of cells (**f**). We observed no changes in dendritic length (**g**), but there was an increase in the total number of spines in the medium spiny neurons of iuGC animals when compared with controls (**h**), as a result of increased number of immature spines (**i**). (**j**) Representative reconstruction of medium spiny neurons of NAcc shell in control and iuGC animals. The NAcc core of iuGC animals also presented volumetric atrophy (**k**) and reduced number of cells (**l**), but preserved dendritic length; spine numbers and mature/immature spine ratio (**m–o**). (**p**) Representative reconstruction of a medium spiny neuron from NAcc core in control and iuGC animals. Data is presented as mean ± s.e.m. CONT, controls; NE, norepinephrine; EPI, epinephrine; 5-HT, serotonin. $^{*}P<0.05$, $^{**}P<0.01$,*** $P<0.001$.

NAcc is seemingly most sensitive to the effects of prenatal GC.

Extending our previous finding that prenatal GC treatment leads to reduced neuronal proliferation in the NAcc,[12] we now report that iuGC results in volumetric atrophy (Figure 2e, shell: $t=4.340$, $P=0.0025$; Figure 2k, core: $t=5.906$, $P=0.0004$) and a reduction of total cell numbers in both the shell and



core divisions of the NAcc in iuGC adult animals (Figure 2f, shell: $t=3.018$, $P=0.0166$; Figure 2l, core: $t=3.760$, $P=0.0055$). Subsequent 3D morphological analysis of dendrites and spines showed that whereas prenatal GC did not influence dendritic lengths of neurons in the NAcc (Figure 2g and m), the treatment produced significant increases in the number of spines within the shell (Figure 2h; $t=3.775$, $P=0.0069$), but not the core division (Figure 2n). The increase in spine number was accompanied by a significant increase in the relative number of immature spines in the shell (Figure 2i; $t=3.108$, $P=0.017$), which, presumably, serve to compensate for the loss of cells in the NAcc and for the reduced amounts of DA reaching the NAcc from the VTA. Notably, although iuGC treatment was associated with increased total spine numbers in the VTA, the treatment did not alter the ratio of immature to mature spines in this region (Supplementary Figure S4). These morphological data, together with the neurochemical data described above, suggest a link between a hypodopaminergic state in the NAcc and the behavioral phenotype observed in animals exposed to prenatal GC.

Altered expression of DA receptor 2 *(*Drd2*) is associated with differential methylation of* Drd2 *gene in iuGC-treated animals*

We next used quantitative reverse-transcription PCR and immunoblotting to identify molecules that might be responsible for the observed behavioral, morphological and neurochemical phenotypes. Expression levels of the mRNAs encoding the GC receptor and corticotropin releasing factor receptors 1 and 2 (all implicated in the neuroendocrine adaptation to stress as well as in drug-seeking behavior[1]), did not differ between controls and iuGC subjects (Supplementary Figure S5). Likewise, no significant differences were found in the expression levels of the synaptic plasticity-related genes *Bdnf*, *synapsin-1*, *Cdk5*, *Creb* and *NCAM* (Supplementary Figure S5). However, there was a significant upregulation of *Drd2* mRNA (Figure 3a; $t=2.764$, $P=0.028$) and DRD2 protein (Figures 3b and c; 35 kDa precursor, $t=3.740$, $P=0.0028$; 47kDa isoform, $t=3.372$, $P=0.005$; 72 kDa glycosylated DRD2, $t=2.177$, $P=0.050$) in the NAcc of iuGC animals. Prenatal GC exposure did not influence either *Drd1* or *Drd3-5* mRNA expression levels (Figure 3a) or the levels of DRD1 protein (50 kDa and glycosylated 74 kDa isoforms; Supplementary Figure S5). In the VTA of iuGC animals, *Drd5* levels were downregulated (Supplementary Figure S5), but the expression of other DA receptors was unchanged (data not shown).

Strikingly, repeated exposure to morphine and ethanol in prenatal GC-treated adult rats led to a significant decrease in the expression of *Drd2* mRNA in the NAcc (Figure 3d; morphine: $t=2.346$, $P=0.043$; ethanol: $t=3.330$, $P=0.0021$). As recent studies reported that psychostimulant treatment induces epigenetic changes in the NAcc,[31–33] we next analyzed the pattern of methylation (strongly correlated with transcriptional repression) in a conserved (human and rodent) CpG island within the *Drd2* gene, covering part of the promoter region and exon 1 (Figure 3e). Our analysis shows that whereas the general DNA methylation profile did not differ between controls and iuGC subjects under basal conditions, overall methylation of the CpG island was significantly increased after chronic morphine administration in adult iuGC-treated animals (Figure 3f–h; $t=3.085$, $P=0.0215$). These changes in DNA methylation are consistent with the finding that *Drd2* expression is downregulated after morphine treatment (Figure 3d). Further, the observation that voluntary ethanol consumption (Figure 3d) also downregulates *Drd2* suggests *Drd2* DNA methylation as a potentially important mechanism in response to substances of abuse.

Restoration of DA levels reverts the molecular, cellular and behavioral phenotype of iuGC animals

The results presented up to this point indicate a strong association between the hypodopaminergic state that prevails in the NAcc of iuGC-exposed subjects and their likelihood to seek drugs of abuse. We next examined whether the phenotype produced by iuGC could be rescued using a simple pharmacological approach. To this end, we administered the DA precursor L-dopa (together with carbidopa to prevent peripheral degradation) for 3 days. This treatment regimen resulted in concomitant increases in DA levels (Figure 4a; $F_{(3,21)}=23.79$, $P<0.0001$) and correspondingly, decreases in *Drd2* expression (Figure 4c; $t=2.982$, $P=0.038$) in the NAcc of controls and iuGC-treated animals. Interestingly, the dynamic *Drd2* response to morphine was normalized after restoration of DA in the NAcc by L-dopa treatment, with iuGC-treated and control animals showing similar patterns of *Drd2* mRNA expression (Figure 4c) and *Drd2* promoter methylation (Figure 4d–f). Interestingly, the neurochemical adjustments induced by L-dopa were accompanied by signs of structural plasticity in the NAcc. These were particularly marked in the core division of the NAcc, where L-dopa-treated animals displayed increased dendritic lengths (more pronounced in iuGC-exposed animals; Figure 4j; $F_{(3,12)}=4.587$, $P=0.023$) and spine numbers (Figure 4k; $F_{(3,12)}=10.01$, $P=0.0014$), though the type of spines were similar between the two groups (Figure 4l). In contrast, increased spine numbers was the only noticable morphological change observed in the NAcc shell (Figure 4h; $F_{(3,10)}=14.86$, $P=0.0005$).

Remarkably, acute (3 days) L-dopa treatment also reversed the vulnerability of iuGC-exposed animals to drug-seeking behaviors, in both contingent ($t=1.851$, $P=0.101$) and non-contingent ($t=0.0192$, $P=0.985$) paradigms (Figures 4m and n), and rescued the hyperlocomotor phenotype displayed by iuGC-treated animals after morphine administration (Figure 4o; $t=2.292$, $P=0.05$). Reversal of these behaviors by

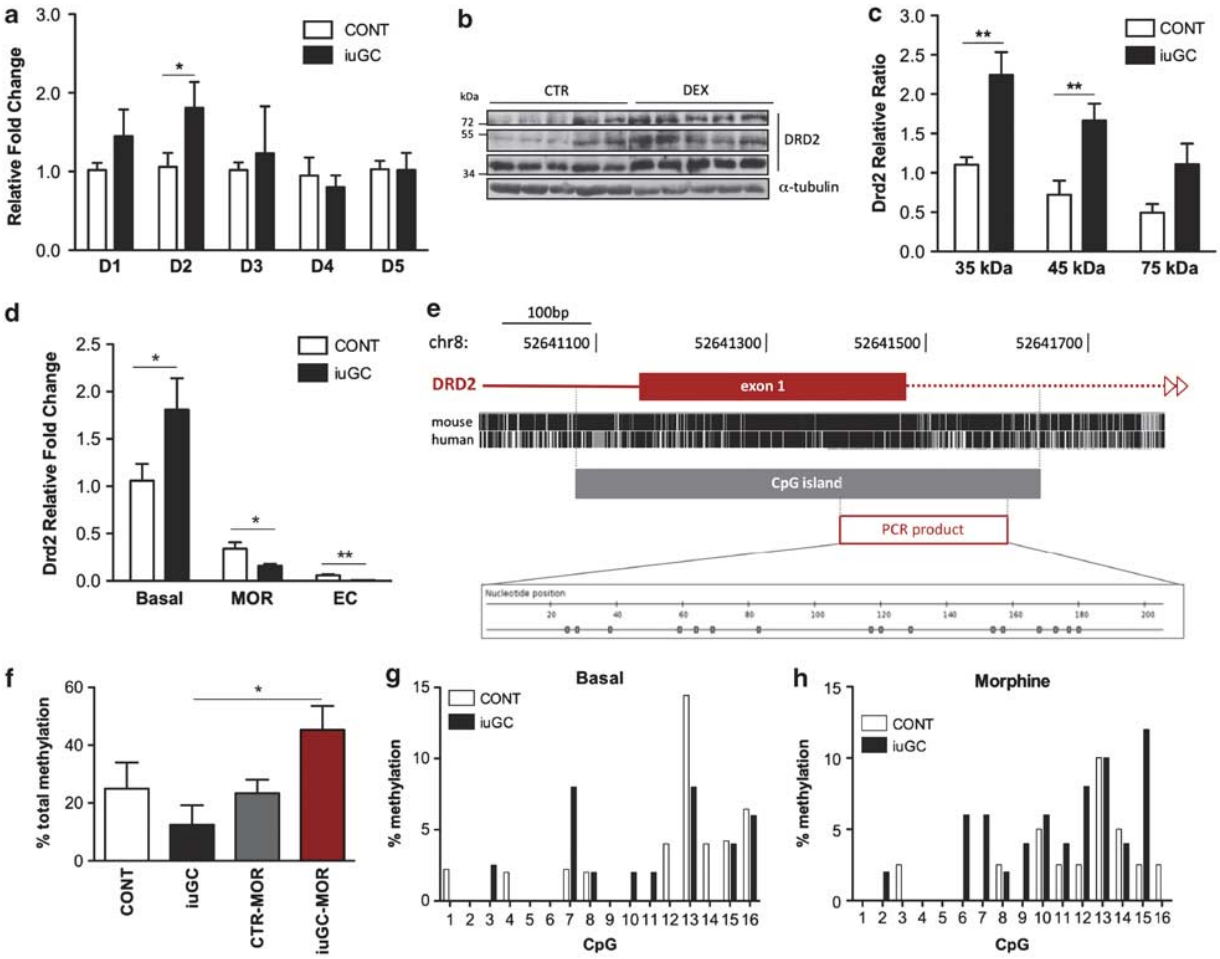


Figure 3 Impaired *dopamine receptor 2* (*Drd2*) response in *in utero* glucocorticoid-exposed (iuGC) animals under basal conditions and after exposure to substances of abuse. (**a**) *Drd2* mRNA expression was augmented in iuGC animals when compared with controls, but no changes were found in the expression of other dopamine receptors. (**b**) Representative immunoblot of DRD2 in five control and five iuGC animals. The levels of the putative DRD2 percursor (35 kDa), the non-glycosylated form (~50 kDa) and the glycosylated receptor (74 kDa) were higher in iuGC animals (**c**). (**d**) Although in a basal situation, *Drd2* was upregulated in iuGC animals, after four injections of morphine (MOR) or 15 days of ethanol consumption (EC), the levels of this receptor were significantly lower in iuGC animals when compared with controls. (**e**) Scheme of the rat *Drd2* CpG island that covers part of the promoter, and exon 1 and respective amplicon with the 16 potential methylation sites are marked (small squares). Also shown is the sequence conservation in humans and mouse (chr8: rat chromosome 8; bp: base pairs). (**f**) Percentage of total *Drd2* CpG methylation in the NAcc of control and iuGC animals revealed a trend for a reduction in the methylation pattern of *Drd2* CpG island in basal conditions, but in opposite pattern after exposure to morphine. (**g**) Percentage of methylation of each dinucleotide in the *Drd2* CpG island in a basal situation. (**h**) After drug exposure, iuGC animals presented an increase in the methylation status of several dinucleotides. Data is presented as mean ± s.d. CONT, controls; $*P<0.05$, $**P<0.01$.

acute L-dopa administration however proved to be only transient; the reversal was not sustained when animals were tested 3 weeks after the last dose of L-dopa (Supplementary Figure S6). On the other hand, when the L-dopa treatment regimen was extended to 3 weeks, reversal of the behavioral, morphological and molecular anomalies associated with a hypodopaminergic state was observable for at least 3 weeks after discontinuation of the drug (Supplementary Figure S6).

Discussion

Work over the last two decades has identified the dopaminergic mesolimbic 'reward pathway,' of which the NAcc is a crucial component, as essential for drug-seeking behaviors.[13,14,34,35] The central role of DA released into the NAcc in the generation of enhanced feelings of pleasure and satisfaction[15] and, thus, in the timing of the initiation of response patterns (e.g., drug-seeking behavior) within the frontocortico-

striatal loop,[36] is well established. Current views suggest that repetitive exposure to drugs of abuse evolve from goal-directed behaviors into habit-based actions.[37,38] We previously demonstrated that stress, associated with increased GC secretion, alters the structure of the corticostriatal loops and steers the development of instrumental behavior into habitual behavior.[39] The present demonstration of GC-induced programming of the structure and function of the NAcc provide, on the other hand, new insights into the mechanisms that underlie the transfer of conditioned behavior to instrumental behavior. Notably, the NAcc (the core in particular) is a crucial determinant of the efficiency of response-outcome associative learning[40] and thus, of the rewarding effects of drugs of abuse;[34] the NAcc modulates motivational drive ('wanting of a reward') and thus, drug-craving. In all these processes, DA seems to have an essential role.

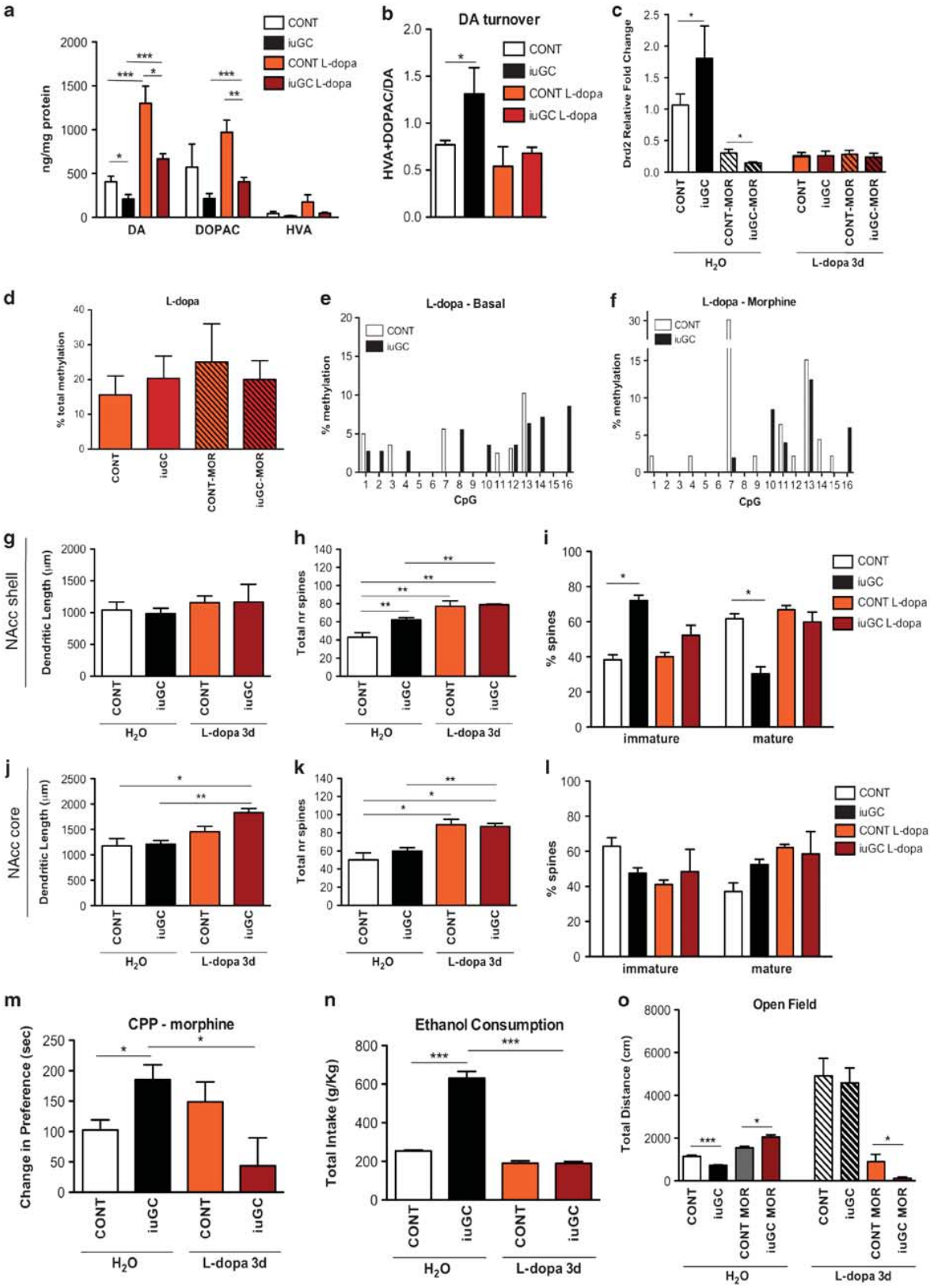
An intricate relationship between stress, the GC released in response to stress, and dopaminergic tone in the regulation of vulnerability to drug and substance abuse has been suggested.[1,5,14,26,41] Stress and drugs of abuse appear to activate dopaminergic synapses in a similar manner,[41] culminating in DA release in the NAcc.[1,4,42] Stress induces sensitization to the psychomotor effects of a number of drugs of abuse and GC have been shown to have an essential role in this process.[1] Specifically, GC are known to modulate the reinforcing properties of drugs and, in fact, have positive reinforcing properties of their own.[43] Adding a new perspective, the present study demonstrates that iuGC triggers an impoverishment in dopaminergic inputs and DA levels in the NAcc, leading to increased drug-seeking behavior in adulthood; notably, hypodopaminergic status is a hallmark of the 'addicted brain.'[44,45] Associated with their lower intra-NAcc levels of DA, animals exposed to prenatal GC expressed more *Drd2* in the NAcc, potentially indicating a compensatory mechanism in this structure. The finding that morphine and ethanol downregulated *Drd2* expression is consistent with the DA-releasing abilities of these substances. The fact that this downregulation is more pronounced in iuGC subjects most likely reflects receptor hypersensitivity due to the hypodopaminergic state previously induced by iuGC.

The regulation of *Drd2*, implicated in different phases of addiction, is seemingly complex;[44] although the short DRD2 isoform interacts with DA transporters and functions as a presynaptic autoreceptor to regulate dopaminergic tone, the long DRD2 isoform is largely localized in postsynaptic targets and mediates the effects of psychostimulants.[46] The present study reveals that vulnerability to substance abuse depends on the dynamic range response of *Drd2* to increased DA release in the NAcc, rather than simply on the expression of *Drd2* at a given time point. Such dynamic regulation is likely to depend on different levels of transcriptional control.

Epigenetic mechanisms are being increasingly implicated in the stable programming by early life events of a spectrum of psychopathological states, including anxiety and depression,[29] impaired cognition[47] and drug abuse,[31–33,48,49] and transient epigenetic modifications have been shown to underlie neural processes such as learning and memory.[50] Such epigenetic changes could imprint dynamic environmental experiences on the unchanging genome, resulting in stable and adaptive alterations in the phenotype. Our results demonstrate that exposure to high GC levels during uterine development increase the risk of drug-seeking behavior in association with altered methylation status of a conserved CpG island in *Drd2* gene and therefore, interfering with the dynamics of *Drd2* expression. Further, they show that repeated administration of morphine to iuGC animals results in marked epigenetic modifications of the *Drd2* gene promoter. These modifications, together with the induced hypodopaminergic state in iuGC-exposed animals, may be considered as key mechanisms that underpin increased susceptibility to drug abuse on one hand, and the dysregulated *Drd2* response to drugs of abuse on the other.

Intriguingly, we found that reduced levels of *Drd2* expression are not necessarily coupled to hypermethylation of *Drd2* gene. Although *Drd2* expression was downregulated after morphine administration in

Figure 4 Restoration of dopamine (DA) levels by L-dopa reverts the molecular, structural and behavioral phenotypes of *in utero* glucocorticoid (iuGC) animals. (**a**) Acute (3 days) treatment with L-dopa increased DA levels in the nucleus accumbens (NAcc) of both experimental groups; although iuGC animals still exhibited less DA than controls. In fact, iuGC animals given L-dopa presented DA levels similar to those of controls without treatment. (**b**) No differences were found in DA turnover after L-dopa treatment in iuGC animals. (**c**) *Dopamine receptor 2* (*Drd2*) expression was diminished after L-dopa treatment both in a basal situation and after morphine exposure (values normalized to controls given water). (**d**) L-dopa treatment did not change *Drd2* methylation status in a basal situation (**e**), but was able to revert the increased methylation in iuGC animals after morphine exposure (**f**). L-dopa supplementation had no significant effect on NAcc shell dendritic length (**g**), but triggered an increase in the number of spines, albeit similarly in control and iuGC animals, and reverted the altered ratio of mature to immature spines observed in iuGC animals (**h** and **i**). (**j**) In contrast, L-dopa treatment increased dendritic length in the NAcc core of both groups. An increase in the number of spines was also observed in both groups with no changes in the type of spines (**k** and **l**). (**m**) L-dopa treatment reverted the higher vulnerability of iuGC animals to morphine-induced CPP and also reverted the ethanol preference displayed by these animals (**n**). (**o**) In agreement, the higher locomotor pattern after morphine displayed by iuGC rats was completely reverted by L-dopa treatment. No differences were found in the locomotion between L-dopa treated control and iuGC animals in a basal situation. Data is presented as mean ± s.e.m. CONT, controls; MOR, after morphine injection 10 mg kg^{-1}; 3d: 3 days; $^{*}P<0.05$, $^{**}P<0.01$,$^{***}P<0.001$.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Total Intake (g/Kg)
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both control and iuGC animals, *Drd2* methylation was observed to a greater extent in the iuGC group. This observation suggests that DNA methylation is not the sole mechanism involved in transcriptional repression of *Drd2* gene. Consistent with this, recent studies have demonstrated interdependence and cooperation between DNA methylation and histone modifications in the regulation of gene silencing and activation.[51] More extensive studies are needed to decipher the precise mechanisms underlying the 'epigenetic potential' of iuGC animals, namely the complex regulation of *Drd2* gene expression, which facilitates adaptation to specific physiological states and demands.

In exploring whether the dynamic epigenetic mechanisms that regulate susceptibility to drug-seeking behavior can be exploited in a therapeutic context, we found that systemic administration of L-dopa reverts drug-seeking behavior in iuGC-treated animals. The latter occured in association with morphological plasticity and significant decreases in *Drdr2* expression levels in the NAcc. Accordingly, we suggest that susceptibility to drug-seeking behavior by iuGC exposure results from the sequential depletion of DA, upregulation of *Drd2* and synaptic impoverishment of dopaminoceptive neurons in the NAcc (Supplementary Figure S7). In this scenario, when DA levels are stimulated by substances of abuse, increased methylation of the *Drd2* gene results in downregulation of *Drd2* expression albeit only in iuGC animals. Strikingly, restoration of DA in the NAcc of iuGC-treated animals also normalizes their *Drd2* responses to subsequent morphine and ethanol exposure, a finding that most likely underlies the above-mentioned reversion of drug-seeking behavior. If translatable to humans, our findings suggest that a simple reinstatement of dopaminergic homeostasis may be sufficient to control addictive behaviors in vulnerable individuals.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Molecular Psychiatry website (http://www.nature.com/mp)

Appendix 3

Mesquita AR, Wegerich Y, Patchev AV, **Oliveira M**, Leão P, Sousa N, Almeida OF

Glucocorticoids and neuro- and behavioural development

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Glucocorticoids and neuro- and behavioural development

Ana Raquel Mesquita [a], Yvonne Wegerich [b], Alexandre V. Patchev [b], Mario Oliveira [a], Pedro Leão [a], Nuno Sousa [a], Osborne F.X. Almeida [b,*]

[a] Life & Health Sciences Research Institute (ICVS), University of Minho, 4710-057 Braga, Portugal
[b] Max Planck Institute of Psychiatry, Kraepelinstrasse 2–10, 80804 Munich, Germany

Keywords:
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Depression
Glucocorticoid
Postnatal
Prenatal
Neurodevelopment
Stress

SUMMARY

Epidemiological evidence links exposure to stress hormones during fetal or early postnatal development with lifetime prevalence of cardiac, metabolic, auto-immune, neurological and psychiatric disorders. This has led to the concept of 'developmental programming through stress'. Importantly, these effects (specifically, hypertension, hyperglycaemia and neurodevelopmental and behavioural abnormalities) can be reproduced by exposure to high glucocorticoid levels, indicating a crucial role of glucocorticoids in their causation. However, there can be important differences in outcome, depending on the exact time of exposure, as well as duration and receptor selectivity of the glucocorticoid applied. The mechanisms underlying programming by stress are still unclear but it appears that these environmental perturbations exploit epigenetic modifications of DNA and/or histones to induce stable modifications of gene expression. Programming of neuro- and behavioural development by glucocorticoids and stress are important determinants of lifetime health and should be a consideration when choosing treatments in obstetric and neonatal medicine.

1. Introduction

Endogenous corticosteroids have a wide spectrum of physiological functions throughout early development, including the regulation of surfactant production by the lungs, salt-water homeostasis, lipid and carbohydrate metabolism, muscle growth and development, immune responses[1] and, as will be discussed, neuro- and behavioural development. Exogenous corticosteroids are indicated in obstetric and pediatric care and are widely used in childhood to treat allergic and/or dermatological symptoms. Corticosteroids are classified as mineralocorticoids or glucocorticoids (GCs), given their respective contributions to ion and water transport in the kidney or in the mobilisation of glucose, respectively. Aldosterone is the prototypic endogenous mineralocorticoid, while cortisol and corticosterone represent the major circulating GCs in humans and rodents, respectively.

Adrenal production of GCs and mineralocorticoids is governed by the secretion of pituitary adrenocorticotropin, controlled by corticotropin-releasing hormone and arginine vasopressin neurons within the paraventricular nucleus (PVN) of the hypothalamus. This top-down sequential activation of the adrenal cortex is tightly regulated through the negative feedback actions of GCs at the pituitary and hypothalamus,[2,3] as well as in higher brain centres, such as the hippocampus and prefrontal cortex (PFC).[4] The hypothalamic–pituitary–adrenal (HPA) axis, like most physiological processes, displays a robust circadian rhythm of activity, but its activation upon perception of a threatening physical, physiological or psychological stimulus (stress) plays a particularly important role in survival. Noxious stimuli occur unpredictably, often at times of the day when the HPA axis is at 'rest'. Mounting an adequate and balanced behavioural and physiological response to adverse stimuli demands finely tuned homeostatic mechanisms that can go awry (transiently or in a protracted fashion) and lead to various brain and other pathologies.

While recent evidence suggests that corticosteroids may produce rapid non-genomic cellular effects[5] via putative membrane-bound receptors,[6] corticosteroids are classically considered to exert their biological action through two ligand-activated transcription factors, mineralocorticoid receptors (MRs) and glucocorticoid receptors (GRs). Ligand binding results in the translocation of the receptor–ligand complexes to the nucleus, where they bind to glucocorticoid response elements (GREs) in the promoter region of target genes to influence gene transcription. Mapping studies have revealed that whereas GRs are ubiquitously distributed, MRs have a more discrete distribution; for example, in the brain MR expression is mainly restricted to one hypothalamic nucleus (the PVN), certain hippocampal subfields and the septum. These patterns of distribution, as well as the pharmacological

* Corresponding author. Tel.: +49 89 3062216; fax: +49 89 306246.
E-mail address: osa@mpipsykl.mpg.de (O.F.X. Almeida).

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profiles of each receptor, to some extent explain the wide spectrum of physiological and behavioural homeostatic functions that are subject to regulation by corticosteroids. Nevertheless, certain enigmas still surround how specificity of actions may be attained given that hippocampal neurons, for example, co-localise both receptors, share presumably identical GREs, and bind the same ligand (cortisol/corticosterone) in the brain; moreover, MRs and GRs differ in affinity by only some 10-fold.[7] Recent attempts to examine whether nuclear co-regulatory proteins may be differentially recruited by the two receptors provided some promise for resolving this important issue.[8,9]

There are convincing links between exposure to GC during early life and the subsequent development of metabolic syndrome (a cluster of cardiovascular risk factors including hypertension, insulin resistance and hyperlipidaemia)[10,11] as well as susceptibility to auto-immune[12] and neurological[13] and psychiatric disorders.[14] According to the increasingly popular concept of developmental programming through stress and GCs, stimuli or insults that occur during critical periods of development can permanently alter tissue structure and function, producing effects that may persist throughout life. The shaping of brain and other physiological functions through endogenous and exogenous signals (stress, GC) at critical periods of development may be compared to the well-known 'organisational' effects of sex steroids during sexual differentiation of the brain.[15,16]

2. Programming of neuro- and behavioural development by glucocorticoids during early life

2.1. Antenatal period

The placenta and most fetal tissues express GRs from mid-gestation onwards.[17] The expression of MRs is more restricted and, at least in rodents, is only detectable during late gestation.[18] In the fetus, steroid hormones are typically involved in organ development and maturation (e.g. lungs, heart, liver, gut and kidneys) and their action during this time has long-term (organisational) effects on the function of the organ in later life. As exogenous GCs can accelerate these processes, they are used therapeutically in preterm labour as well as in the perinatal period, in particular to help lung maturation.[19] Given their small size and lipophilicity, GCs can access the brain; the magnitude of their damaging effects in the brain depends largely on the particular stage of development when exposure occurs,[20] indicating that there are 'critical windows' of sensitivity to the detrimental effects of GCs on brain tissue; again, these windows of sensitivity may be analogous to those described for sexual differentiation of the brain through the actions of sex steroids.[15]

Spatio-temporal fine-tuning of GC levels during ontogeny is mandatory for appropriate organ maturation while avoiding undesirable side-effects. The fetus has only limited capacity to degrade steroids, especially synthetic (xenobiotic) GCs, which means that even low levels of GCs can have prolonged access to sensitive tissues. This can have detrimental effects especially in tissues undergoing rapid proliferation and differentiation, e.g. the brain. Here, it should be noted that although endogenous GC can bind both GRs and MRs, their effects (at least in the brain) may be regulated in a rheostatic manner, depending on their relative occupation of each receptor[21,22]; on the other hand, the typical GCs used in obstetrics and neonatology (e.g. dexamethasone, betamethasone) tend to be have a greater affinity for GRs which, as will be discussed later, appear to mediate many of the undesired actions of GCs in the brain. In this context, it should be mentioned that fetal GC levels are much lower than those found in the maternal circulation, as a consequence of the action of the enzyme 11β-hydroxylase 2 (11β-HSD2), which regulates the conversion of active GCs (cortisol and corticosterone) into their inactive 11-keto metabolites (cortisone and 11-dehydrocorticosterone, respectively).[23,24] 11β-HSD2 is present in the syncytiotrophoblast, the site of maternal–fetal exchange, where it is well-positioned to serve as the barrier for GC transfer and, consequently, to protect the fetus from the adverse effects of excessive maternal GC. However, this mechanism does not provide complete protection; studies in rats and humans indicate considerable variations in placental 11β-HSD2 activity towards the end of pregnancy,[25] possibly explaining why stress-induced increases in GC secretion in the mother may surmount the placental barrier and produce deleterious effects in the developing fetus. The fetal brain transiently expresses high levels of 11β-HSD2; levels decline after mid-gestation in a manner that varies from one brain area/neuronal population to another.[26]

Experimentally, the effects of increased GC load on the fetal brain have been studied by exposing pregnant rodent dams either to exogenous natural or synthetic GCs or to various stressors (e.g. repeated immobilisation) from the last week of gestation (typically, from embryonic day 14 [E14], with delivery at E21/22, corresponding, approximately, to the second trimester of human pregnancy).[27] It should be noted however, that differences in maternal care[28] may be an important confounding factor in the interpretation of results involving such paradigms, especially those pertaining to brain maturation and eventual psychopathology.

Synthetic GC (e.g. dexamethasone) administration to pregnant rat or mouse dams is frequently used to experimentally increase the foetus' exposure to GC. If at all, exogenous GCs are poor substrates for 11β-HSD2, and they therefore readily cross the placenta.[29] Rats receiving acute dexamethasone (selective GR agonist) during the last third of pregnancy display an anxious phenotype and signs of impaired GC negative feedback in adulthood; such signs are not seen in animals receiving corticosterone (binds to MR and GR) at an equivalent dosage, indicating the differential effects of GR vs MR occupation in the programming of behavioural functions.[30] Interestingly, not all behavioural domains are differently affected by corticosterone and dexamethasone, suggesting that the neurochemical and anatomical substrates which mediate anxiety behaviour might be differentially sensitive to corticosteroids at different developmental stages due to the relative temporal availability of GR and MR in a specific brain area.[31] Prenatal stress affects mood and emotional behaviour in a manner similar to that observed with exogenous GC: as adults, animals that experienced stress in utero display depression-like[32] and hyper-emotional (anxious) phenotypes.[33] The underlying anatomical and neurochemical correlates of these altered behaviours remain ambiguous, but a variety of neurotransmitter systems and signalling cascades can be expected to be affected by antenatal GC exposure. Interestingly, some of the stress-induced behavioural symptoms can be normalised with antidepressants,[34] a finding that matches reports that antenatal GC exposure reduces 5-HT1A receptor expression,[35] a phenomenon causally related to depression-like behaviour.

Apart from these well-known behavioural effects of inappropriate antenatal GC exposure and their clinical projections for the pathogenesis of mood and anxiety disorders, accumulating evidence suggests that other behavioural functions are also subject to GC-mediated programming. Prenatal stress reportedly also results in reduced exploratory behaviour, presumably a reflection of their reduced motivational drive.[36] Locomotor behaviour has been frequently assessed in animals that were exposed to prenatal stress or exogenous GC, but the findings for this behavioural endpoint have been somewhat controversial. Locomotor activity after acute adult pharmacological or environmental challenges in prenatally stressed or GC-treated animals reflects the malprogramming effects of stress/GCs on motivational and appetitive functions. For instance, amphetamine-stimulated locomotion was

found to be accentuated in adult rats that had been stressed during prenatal life, but interestingly, these effects were only manifest when these animals were exposed to an acute stressor in adulthood.[37] These patterns have been attributed to stress-induced alterations in catecholaminergic systems.[38] The behavioural patterns observed in animals are similar to those seen after acute amphetamine application in unmedicated schizophrenic patients; striatal dopaminergic transmission is disturbed in such patients who are characterised by their impaired ability to discriminate between relevant (signal) and irrelevant (noise) signals in the prepulse inhibition (PPI) test.[39] Importantly, there are strong links between maternal stress in human pregnancy and the incidence of schizophrenia in the offspring.[40]

Addictive behaviour represents another behavioural domain that seems to be susceptible to GC programming during early life. An increasing number of studies indicate that the experience of high GC levels during late gestation can predispose individuals for developing drug dependence in adolescent and adult life. In trying to identify the neuromorphological correlates of these behavioural changes, we recently evaluated the impact of prenatal GC exposure on the structure and function of the nucleus accumbens (NAcc), a crucial relay point in the limbic circuitry of reward. We observed that the volume and number of cells in the NAcc were significantly reduced in the adult progeny of dams treated with dexamethasone during their last trimester of pregnancy; these findings were paralleled by reduced rates of cell proliferation in the ventral tegmental area (VTA, an area that sends dopaminergic projections to the NAcc) as well as in the shell and core of the NAcc. In addition, prenatal GC treatment resulted in reduced dopaminergic innervation of the NAcc.[41] Previously, major structural reorganisation of the NAcc and VTA were described during late gestation[42]; the data from Leão et al.[41] therefore indicate that GC may interfere with this process, producing structural and neurochemical alterations that predispose to drug-seeking behaviour.

Most psychiatric disorders (depression, anxiety and schizophrenia) associated with gestational stress are characterised by cognitive impairments. The hippocampus, which is critical for learning and memory, has an abundant population of corticosteroid receptors.[21] A very robust finding in the literature is that hippocampal volumes and numbers of neurons and synaptic contacts are reduced in prenatally stressed rats.[43] Moreover, the adult offspring of mothers subjected to stress during gestation show significant impairments in spatial learning[44] and long-term potentiation, the electrophysiological correlate of memory[45]; these physiological and behavioural impairments most likely occur secondarily to the above-mentioned morphological changes. Nevertheless, and despite many published studies on the association between antenatal stress and reduced cognitive performance in animals, we and others failed to observe deficits in learning ability and memory in the offspring of rats born to mothers that were either stressed or exposed to exogenous corticosteroids during pregnancy.[30,46] Such discrepancies could arise from the quality of the postnatal environment (including health status of the mother); for example, rats born to dexamethasone-treated mothers, but reared by vehicle-treated mothers, were found to display normal spatial learning and enhanced sensitivity to GC negative feedback, compared with animals born to, and raised by, dams that were given dexamethasone during pregnancy.[47] Thus, the early postnatal environment seems to play a critical role in the determination of behavioural and neuroendocrine outcomes.[48] Importantly, these findings also point to the reprogrammability of events initiated during prenatal life by postnatal experience; this reprogramming will, presumably, be compromised by any stressful experience after birth, and it is likely to be limited to periods after birth when neuronal plasticity (within and between brain regions) is still dynamic.

2.2. Neonatal period

Behaviour is critically dependent on brain development, including appropriate neuron numbers and synapses, as well as connectivity between different brain areas. Despite this, there is a notable paucity of information available about how stress and GC experience during early postnatal life affect behaviourally relevant brain structures. Early studies described hippocampal atrophy following exposure to stress or exogenous corticosteroids during early life. This atrophy can be accounted for by dendritic/axonal changes,[49] and a decrease in neuronal number[50]; the latter could be a consequence of changes in neuronal survival and plasticity as a result of reduced neurogenesis and increased apoptosis[51] and altered neurotrophin expression.[52] Since the hippocampus plays a critical role in learning and memory and connects to other brain regions that are involved in the regulation of mood and emotionality, as well as neuroendocrine regulation, these permanent changes can be expected to have a major impact on behaviour and physiology. One such region is the PFC which is implicated in the regulation of cognition, mood, emotion and motivation. Braun and collaborators found that early life stress results in a decrease in dendritic spine density and number of pyramidal neurons in the anterior cingulate cortex, and an increase in these measures in the dorsal part of the PFC,[53] although the functional significance of these findings remains to be elucidated.

The programming effects of stress in early postnatal life (first week of life in rodents corresponds, roughly, to late pregnancy in primates) have been extensively assessed. Most studies employed the so-called maternal separation (MS) stress paradigm, which is based on interference with the mother–infant interaction. Even though phenotypic outcomes are not always easy to compare between studies, possibly due to subtle differences in experimental protocols (e.g. frequency and duration of MS, age at which the paradigm is first applied, level of social deprivation, as well as the quality of the post-separation and post-weaning environments), most studies report behavioural aberrations that may be related to many aspects of mental illness in humans, such as mood and anxiety disorders as well as schizophrenia. For instance, Ellenbroek and colleagues[54] showed that separation of rat pups from their mothers for a single 24 h session results in attention deficits and in a disruption of PPI which, as previously mentioned, suggests schizophrenia-like dysfunction. Interestingly, this behavioural phenotype was only expressed after puberty,[54] thus resembling the temporal profile observed for the onset of schizophrenic symptomatology in patients and in line with the concept that the impact of early-life stress on sensorimotor gating depends on the age at which the stress is experienced.[54,55] The mechanisms through which early-life stress induces these behavioural abnormalities remain unclear at present; however, their responsiveness to antipsychotic drugs[54] indicates hypersensitivity in the dopaminergic system. In addition, MS-stressed rats (A.R. Mesquita, unpublished data) and mice[56] display, as adults, signs of increased learned helplessness, a sign of depressive-like behaviour that can be prevented by antidepressant administration at the end of the MS paradigm.[57] In these types of experiments too, narrow periods of sensitivity to the deleterious effects of GCs or MS have been observed. However, these 'windows of vulnerability' may differ from one behavioural domain to another. For example, prenatal manipulations of the GC milieu do not influence learning and memory[30]; nevertheless, significant impairments of these measures of cognition have been observed in MS-stressed rats (unpublished data).

Variations in maternal care may serve an important role in determining the behavioural outcome of early-life stress. For instance, Liu et al.[58] found that the learning impairments induced by MS were greater in the adult offspring of animals reared by

poor-caring vs those fostered by good-caring mothers. Similar reports exist with respect to quality of maternal care and MS-induced deficits in sensorimotor gating, mood and anxiety.[59,60]

Besides the behavioural programming described above, early-life events are known to affect somatic well-being on measures of body mass and growth. However, their impact on the timely reaching of critical milestones has not been studied extensively. Recent studies in our laboratory[61] have shown that MS on postnatal days 2–15 delays the acquisition of neurological reflexes that are dependent on vestibular and cerebellar function. On the other hand, MS-treated animals showed earlier eye and ear opening, indicating advanced physical maturation. Similarly, Neal[62] reported that dexamethasone administration during the first postnatal week leads to retardation of acquisition of the above-mentioned neurological reflexes. While some authors have suggested that delayed myelination can account for these neurological deficits,[63–65] we recently found that the serotoninergic system might also be involved[61]; specifically, MS-experienced rats showed increased serotonin turnover in the dorsal raphe nucleus.

It is important to note that the neurochemical bases of MS are poorly understood. Importantly, GCs do not seem to be directly responsible for any of the above-mentioned effects since GCs are not secreted in response to stress in the rodent neonate[66]; this so-called stress hyporesponsive period is confined to about the first two weeks of the rodent's postnatal life. The fact that MS and postnatally administered GCs may lead to similar behavioural and neuroendocrine outcomes, suggests possible redundancy of signals that converge on stress-sensitive neural substrates. Resolution of this issue remains a challenge for future research.

3. Are animal studies translatable into clinical practice?

In its 2001 Mental Health Report, the World Health Organization observed that, 'Contrary to popular belief, mental and behavioural disorders are common during childhood and adolescence. Inadequate attention is paid to this area of mental health'. The Report went on to point out that, 'Mental and behavioral disorders of childhood and adolescence are very costly to society in both human and financial terms. The aggregate disease burden of these disorders has not been estimated, and it would be complex to calculate because many of these disorders can be precursors to much more disabling disorders during later life'. Happily, these concerns are currently being addressed,[67] and some of the results discussed above indicate that the various experimental paradigms and animal models may be relevant to human health.

Glucocorticoids are indicated in obstetric and paediatric conditions, such as infection, connective tissue and allergic disorders, and are commonly used in obstetric practice to accelerate lung maturation in cases of threatened preterm labour; the latter affects up to 10% of pregnancies. While GC therapy makes a significant contribution to the reduction of infant mortality,[68] there is considerable debate about which GC should be used, and according to what regimen, for a particular underlying condition, so as to induce the minimum degree of damage. Only a few studies have assessed the impact of perinatal GC exposure in humans and there is usually sparse follow-up information available. The significant findings that have so far emerged may be summarised as follows: (i) children exposed to dexamethasone and who were born at term, had increased emotionality, unsociability/social withdrawal and general behavioural problems[69] as well as persistent impairments in tests of verbal working memory[70]; (ii) the offspring of women given multiple doses of antenatal GCs because of risk of preterm delivery have reduced head circumferences, and show significantly increased aggressive/violent behaviour and attention deficits.[71] Likewise, maternal stress has been shown to induce long-term programmes in the fetus that prevail at least until middle childhood[72] and, as shown by recent work from Rachel Yehuda's laboratory, children of mothers with post-traumatic stress disorder display altered cortisol levels that are accompanied by signs of behavioural distress during the first nine months of life.[73] In addition, the stress-induced release of striatal dopamine is reduced in subjects that had experienced poor parental bonding, indicating dysregulation of neurotransmitter systems by stressful experiences during early life.[74]

These clinical reports reveal a pattern similar to that observed in experimental animal models, but which itself is insufficient to allow immediate extrapolation to humans. Taken together, they do, however, point to the long-term negative impact that high GC levels can have on the development of the brain and behaviour. However, one should consider that risk–benefit judgements are confounded by the fact that subjects born preterm are already at risk for delayed neurodevelopment. A recent systematic review concluded '… prenatal steroids reduce the occurrence and severity of … health problems in the first few weeks of life …. However, these benefits are associated with a reduction in some measures of weight and head circumference at birth, and there is still insufficient evidence on the longer-term benefits and risks'.[75]

4. Mechanisms of programming by stress and glucocorticoids

Stable alterations of gene expression that are independent of changes of the DNA sequence can be caused by epigenetic mechanisms. Covalent modifications, e.g. through methylation of DNA at cytosine residues of CpG dinucleotides or through various histone modifications, are among the best known of these. Work by Avishai-Eliner et al.[76] and Liu et al.[58] showed that social environmental cues, such as postnatal handling, promote maternal care, resulting in long-lasting increases in GR expression in the hippocampus of rat pups raised by high-caring mothers. Recently, pioneering work in the laboratories of Michael Meaney and Moshe Szyf demonstrated that demethylation at the nerve growth factor1A (NGFI-A, also known as Egr1, zif268 or Krox 24) binding site may underlie the increased expression of GRs; NGFI-A is a key transcription factor in the GR promoter, activating transcription at the GR 1_7 promoter.[77,78] Studies in vitro and in vivo demonstrated that demethylation at the NGFI-A binding site in the rat GR 1_7 promoter is initiated by enhanced serotonin input and activation of 5-HT_7 receptors which, in turn, activate the cAMP/protein kinase A pathway, increasing NGFI-A expression; NGFI-A binding to its recognition element in the 1_7 GR promoter initiates cytosine demethylation and leads to the recruitment of a histone acetylase (cyclic AMP response element-binding protein), an event that could potentially explain enhanced transcription of the GR.[78] Interestingly, cross-fostering studies revealed a direct effect of maternal care, rather than genetic inheritance, suggesting trans-generational transmission of these epigenetically programmed traits.[79] While pointing to mediation through serotonin, it should be mentioned that GCs themselves have been shown to have the potential to induce epigenetic changes in non-neural tissues; for example, chronic treatment of rat hepatoma cells with GC induces fast chromatin remodelling in an enhancer region of the GR target gene tyrosine aminotransferase, an event followed by recruitment of the transcription factor hepatic nuclear factor-3 (HNF3) and slow, but persistent DNA demethylation at GC-responsive units within the enhancer.[80,81]

5. Conclusions

An abundance of data in rodents and humans indicates that exposure to GC at specific time-points during ante- and postnatal development can induce undesired cardiovascular, metabolic, neuroendocrine and behavioural phenotypes in adulthood. The

concept that stress and GCs act by reprogramming the genetically determined phenotype through epigenetic mechanisms is becoming increasingly popular; more intriguing is the evidence that some of these acquired changes may be transmitted across generations. Improvements in our understanding of these latter two mechanisms are a major challenge for researchers in this field; in particular, it will be of interest to discover ways in which human health can be improved by over-writing these changes.

Conflict of interest statement

None declared.

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